National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

THE UNIVERSITY OF ALBERTA

STUDIES OF PLANT GAMMA-GLUTAMYL HYDROLASE

BY

SONG LIN

©

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
AND RESEARCH IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF MASTER OF
SCIENCE

IN
PLANT BIOCHEMISTRY

DEPARTMENT OF BOTANY

EDMONTON, ALBERTA
FALL, 1993

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-88044-9

Canada

THE UNIVERSITY OF ALBERTA
RELEASE FORM

NAME OF AUTHOR: SONG LIN

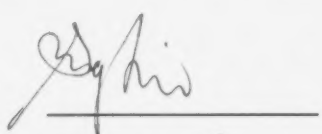
TITLE OF THESIS: STUDIES OF PLANT GAMMA-GLUTAMYL HYDROLASE

DEGREE: MASTER OF SCIENCE

YEAR THIS DEGREE GRANTED: FALL, 1993

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves all other publication rights, and neither the thesis nor any portion thereof may be printed or otherwise reproduced without the author's written permission.



#446, East 10th Ave
Vancouver
British Columbia
V5T 2A1

Date: August 30, 1993

THE UNIVERSITY OF ALBERTA
FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled: STUDIES OF PLANT GAMMA-GLUTAMYL HYDROLASE submitted by SONG LIN in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE IN PLANT BIOCHEMISTRY.

Edwin A. Cossins (supervisor)

Arnost Horak

David Gifford

Michael Hiokman (chairman)

Date: August 30, 1993

TO MY PARENTS

Out of the cradle

endlessly rocking

Out of the mock-bird's throat

the musical shuttle...

--From Walt Whitman's

Leaves of Grass

ABSTRACT

Gamma-glutamyl hydrolase (GGH), (EC 3.4.22.12), hydrolyzes polyglutamyl folates into simple forms. Several animal GGH proteins have been characterized but previous work on plant GGH has mainly centered on studies of relatively crude tissue extracts. In the present study, a GGH protein of *Pisum sativum* was extracted from cotyledons and purified over 45,000-fold. Differential centrifugation of mannitol-buffered tissue extracts showed that GGH activity of the cotyledon tissues was mainly cytosolic with minor amounts detected in the vacuolar and mitochondrial fractions. The activity, based on the hydrolysis of [^{14}C]-PteGlu$_3$, was maximal at pH 6 and associated with a M_r *ca.* 55 kD protein. The major properties of this hydrolase were similar to those reported for the intracellular GGH proteins of mammals. Pea GGH had an endopeptidase action, and required at least the p-aminobenzoyl moiety of the substrates for the hydrolysis of the attached gamma-glutamyl peptide. This plant enzyme was capable of hydrolyzing either [^{14}C]-PteGlu$_3$ or [^{14}C]-H$_4$PteGlu$_3$. However, the hydrolysis of [^{14}C]-PteGlu$_3$ was strongly inhibited by PteGlu$_5$, zinc ions, phenylmethylsulphonylfluoride, p-hydroxymercuribenzoate and 2-mercaptoethanol but not by 2-mercaptomethylglutaric acid (0.04-4 mM), heparin, glutamic acid and bovine serum albumin.

ACKNOWLEDGEMENT

I would like to thank my supervisor, Dr. Edwin A. Cossins for providing such an opportunity for me to study at the University of Alberta, and for his encouragement throughout the course of this program.

I would also like to thank all my friends in the lab. Their help and friendship are much appreciated and will always be warmly remembered. My thanks also go to Ms. Vindy Lin for her excellent secretarial assistance in preparing the manuscript of this thesis, especially in drawing Fig. 5 and 6.

Thanks also to Dr Jesse F. Gregory III, Department of Food Science and Human Nutrition, University of Florida, for sending me related paper in addition to the reprints of his own articles.

I gratefully acknowledge the financial support I received from Dr. Cossins and the Department of Botany. I also acknowledge the travel assistance provided by the Faculty of Graduate Studies and Research. This travel assistance partially enables me to attend the Folic Acid, Vitamin B_{12} and One-Carbon Metabolism Conference, Vermont, U.S.A., and present part of this thesis as a poster.

The research presented in this thesis was funded by a NSERC (the Natural Sciences and Engineering Research Council of Canada) research grant given to Dr. Cossins.

TABLE OF CONTENTS

CHAPTER PAGE

1. INTRODUCTION 1

1-1. Overview of One-Carbon Metabolism 1

1-1-1. Sources of C_1 Units 1

1-1-2. The Interconversion of C_1 Units 4

1-1-3. The Utilization of C_1 Units 5

1-1-3-1. The Methionine Cycle 6

1-1-3-2. The Thymidylate Cycle 7

1-1-3-3. The Purine Cycle 9

1-1-3-4. The Initiation of Protein Synthesis 10

1-2. Folylpolyglutamates: Biosynthesis and Degradation 10

1-3. GGH Activity and Cross-Membrane Transport of Folate 15

2. MATERIALS AND METHODS 18

2-1. Chemicals 18

2-2. Plant Material 18

2-3. Assay of GGH Activity 18

2-4. HPLC Analysis of Folate Products 19

2-5. GGH Extraction and Purification 21

2-6. Isolation of Vacuoles from Pea Cotyledons and Leaves 22

2-7. Isolation of Mitochondria from Pea Cotyledons 24

2-8. Preparation of [^{14}C]-Tetrahydrofolatetriglutamates 27

3. RESULTS 29

3-1. GGH Activity of Pea Cotyledons 29

3-2. Partial Purification of GGH Protein 34

3-3. GGH Stability and Reaction Requirements..... ..... .39
3-4. Kinetic Studies of GGH..... .43
3-5. Inhibitors of GGH Activity..... 43
3-6. Molecular Weight of Pea Cotyledon GGH..... 50
3-7. Analysis of Reaction Products..... 50
3-8. Substrate Specificity of GGH..... 50
3-9. Activity of GGH When Incubated with Protein-Bound Substrates 55
4. DISCUSSION..... ..... .60
4-1. Purification of Pea Cotyledon GGH.... 60
4-2. Major Properties of Pea Cotyledon GGH.. 60
4-3. The Possible Regulation of Pea Cotyledon GGH Activity..... 62
4-4. Some Possible Roles of GGH Activity in Folate and
Antifolate Metabolism ..... .63
4-4-1. Regulation of Folylpolyglutamate Chain Length by GGH Activity .64
4-4-2. Control of Cross Membrane Folate Transport by GGH Activity... .65
4-4-3. GGH Activity and Methotrexate Resistance 67
4-5. Suggestions for Further Study..... ..... .67
5. BIBLIOGRAPHY..... .69

LIST OF TABLES

TABLE #	TITLE	PAGE
1.	Effects of extraction buffer and storage at 4^{o}C on pea cotyledon GGH activity	.30
2.	Changes in GGH activity during pea seedling growth	...32
3.	Intracellular distribution of GGH activity	..33
4.	Purification of GGH activity from 6-day old pea cotyledons	35
5.	The effects of heparin, glutamic acid, BSA and 2-mercaptoethanol on GGH activity	.45
6.	Gamma-glutamyl tripeptide and N-CBZ-phe-ala are not hydrolyzed by GGH protein	58

LIST OF FIGURES

FIGURE #	TITLE	PAGE
1.	General pathway of C_1 metabolism	2
2.	Structure of folylpolyglutamate	11
3.	De novo pathway of folate biosynthesis	13
4.	Separation of folate and non-folate products on DE-52 cellulose column	20
5.	Purification of protoplasts by gradient centrifugation	25
6.	Isolation of vacuoles from protoplast lysate	26
7.	Effect of assay pH on GGH activity	31
8.	Chromatography of GGH protein on DEAE-Sephacel	36
9.	Gel filtration of GGH activity	37
10.	Affinity chromatography on Matrex Green A	38
11.	Chromatography of GGH protein on heparin agarose	40
12.	Effect of assay temperature on GGH activity	41
13.	Time course of GGH product formation	42
14.	Effect of GGH concentration on the hydrolysis of $PteGlu_3$	44
15.	Lineweaver-Burk plot for pea GGH using $PteGlu_3$ as substrate	46
16.	Lineweaver-Burk plot for pea GGH using $H_4PteGlu_3$ as substrate	47
17.	Inhibition of GGH activity by PMSF and Zn ions	48
18.	The effect of MMGA and p-HMB on GGH activity	49
19.	Determination of molecular weight of GGH	51
20.	Determination of folate chain lengths by HPLC	52

21. Hydrolysis of $PteGlu_3$ and $PteGlu_5$ 53

22. Formation of gamma-glutamyl dipeptide after [^{14}C]-$PteGlu_3$ hydrolysis 54

23. Hydrolysis of $pABAGlu_3$ 56

24. The effect of gamma-glutamyl tripeptide, PteGlu and $PteGlu_5$ on the hydrolysis of $PteGlu_3$ 57

25. Effect of DHFR on the hydrolysis of $PteGlu_3$ and $H_4PteGlu_3$ 59

LIST OF ABBREVIATIONS

AICAR	5-phosphoribosyl-5-amino-4-imidazole
AIDS	acquired immuno-deficiency syndrome
BSA	bovine serum albumin
C_1	one-carbon
C_1-THF	one-carbon substituted tetrahydrofolate
DEAE	diethylaminoethyl
DHFR	dihydrofolate reductase
dpm	disintegrations per minute
fMet-tRNA	formylmethionyl transfer RNA
FPGS	folylpolyglutamate synthetase
GAR	5-phosphoribosylglycinamide
GGH	gamma-glutamyl hydrolase
$H_4PteGlu_n$	tetrahydrofolylpolyglutamate
10-HCO-$H_4PteGlu_n$	N^{10}-formyl-$H_4PteGlu_n$
5,10-CH=$H_4PteGlu_n$	N^5,N^{10}-methenyl-$H_4PteGlu_n$
5,10-CH_2=$H_4PteGlu_n$	N^5,N^{10}-methylene-$H_4PteGlu_n$
5-CH_3-$H_4PteGlu_n$	N^5-methyl-$H_4PteGlu_n$
HPLC	high performance liquid chromatography
MMGA	mercaptomethylglutaric acid
MS	methionine synthase
MTX	4-amino-10-methyl-THF (methotrexate)
MTXPG	methotrexate polyglutamate
N-CBZ-phe-ala	N-carbobenzoxyphenylalanyl-alanine
pABA	p-aminobenzoic acid

$pABAGlu_n$	p-aminobenzoyl polyglutamate
PCP	*Pneumocystis carinii* pneumonia
PMSF	phenylmethylsulfonyl fluoride
p-HMB	p-hydroxymercuribenzoate
PteGlu	pteroylglutamic acid (folic acid)
$PteGlu_3$	pteroyltriglutamate
SHMT	serine hydroxymethyltransferase
TS	thymidylate synthase

INTRODUCTION

Folate coenzymes are ubiquitous in every known organism and cell type. One-carbon transfers mediated by folate coenzymes play essential roles in several major cellular processes, including DNA biosynthesis, bacterial and organellar protein biosynthesis, amino acid metabolism, methyl group metabolism and vitamin metabolism (Appling, 1991). The requirement for folate coenzymes for the synthesis of purines and thymidylate has led to the development of a large number of drug therapies that target folate mediated one-carbon metabolism. Antifolate drugs are widely used in the treatment of cancer. Many cytotoxic folate analogs, after entering cells, become polyglutamated, which may affect their inhibitory activities, selectivity, toxicity and duration of action (Kalman, 1990). The effects of antifolate polyglutamation on the cytotoxic efficacy of these drugs can be explained by studies of enzyme affinity and the cellular retention of folylpolyglutamates of different chain lengths, as well as the factors affecting the biosynthesis and degradation of these polyglutamate chains.

1-1 Overview of One-Carbon Metabolism

The series of reactions leading to the transfer of one carbon units to produce important cellular constituents are commonly referred to as one-carbon metabolism (C_1 metabolism). C_1 metabolism plays an important role in several major metabolic pathways (Figure 1). All of these pathways involve folate-dependent reactions and these can be classified into 3 groups as shown in Figure 1. Thus C_1 metabolism includes the generation of C_1 units, the interconversion of C_1 units and the utilization of C_1 units.

1-1-1. Sources of C_1 Units

The major sources of C_1 units include the degradation of serine and glycine. In addition, available formate may be utilized as a precursor of 10-formyltetrahydrofolate.

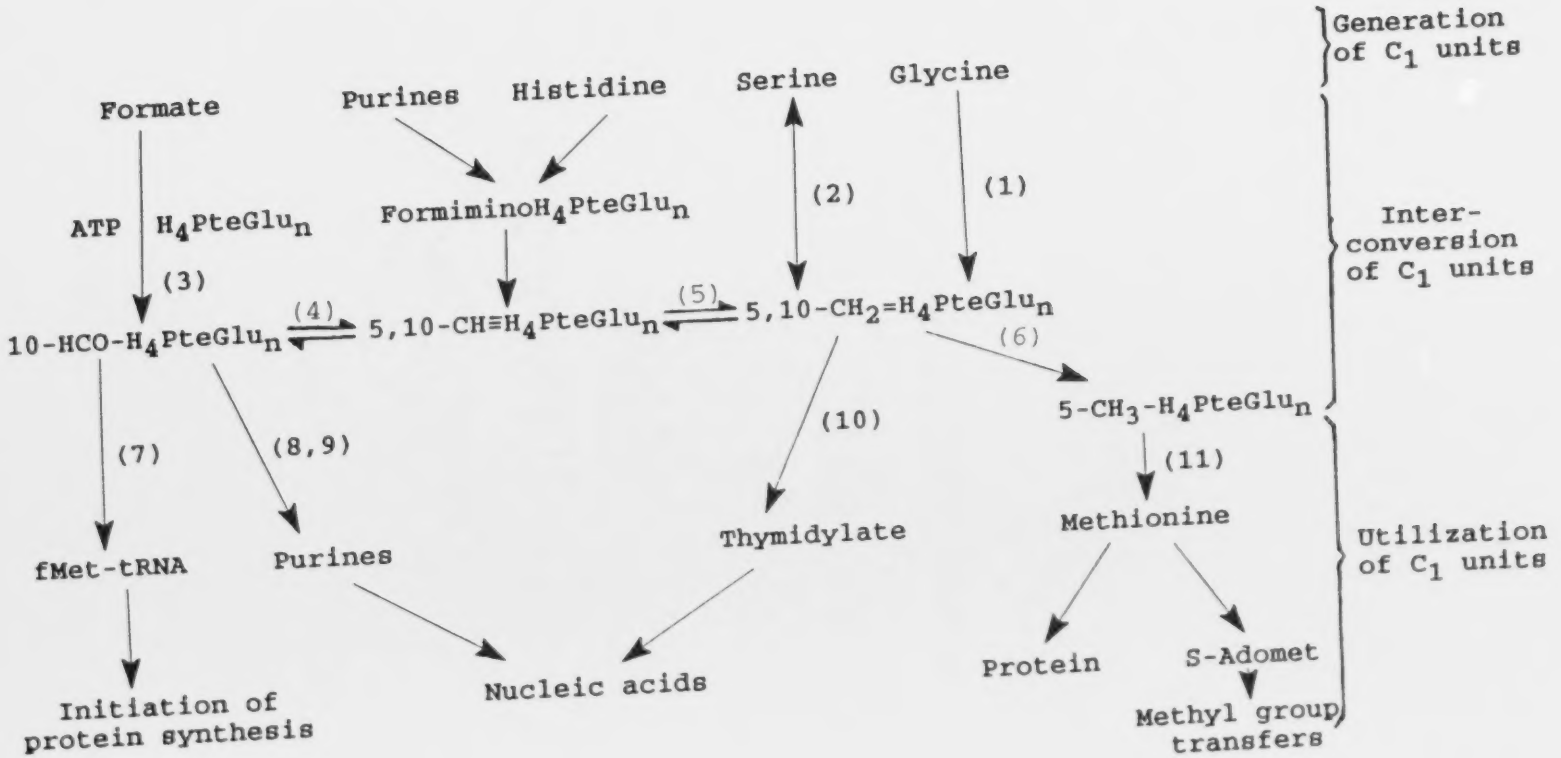


Fig. 1 General Pathway of C_1 Metabolism. Enzymes catalyze the above folate mediated reactions are: (1) Glycine synthase, (2) Serine hydroxymethyltransferase, (3) 10-formyl-tetrahydrofolate synthetase, (4) 5,10-methenyltetrahydrofolate cyclohydrolase, (5) 5,10-methylenetetrahydrofolate dehydrogenase, (6) 5,10-methylenetetrahydrofolate reductase, (7) Methionyl-tRNA transformylase, (8) Glycinamide ribotide (GAR) transformylase, (9) Phospho-ribosylaminoimidazole carboxamide (AICAR) transformylase, (10) Thymidylate synthase, (11) Methionine synthase.

Generally speaking, serine and glycine are quantitatively the most important contributors of C_1 units (MacKenzie, 1984). Futhermore, histidine is a minor source for C_1 units, and it will not be discussed here. The reader is referred to an excellent review by Shane and Stokstad (1984) for more information on this topic.

Serine and glycine are both involved in two folate-dependent reactions that generate 5,10-$CH_2H_4PteGlu_n$. These reactions are catalyzed by glycine synthase, EC 2.1.2.10 (equation 1) and serine hydroxymethyltransferase (SHMT), EC 2.1.2.1 (equation 2).

$$\text{Glycine} + H_4\text{PteGlu} + \text{NAD} \rightleftharpoons 5,10\text{-}CH_2{=}H_4\text{PteGlu} + \text{NADH} + CO_2 + NH_3 \quad (1)$$

$$\text{Serine} + H_4\text{PteGlu} \rightleftharpoons 5,10\text{-}CH_2{=}H_4\text{PteGlu} + \text{Glycine} \quad (2)$$

The glycine synthase of bacteria and that located in the mitochondria of animal and plant cells is a complex of four closely associated proteins designated P-, H-, L-,and T-proteins (Schirch, 1984). The general properties of these subunits has been reported using pea leaf (Neuburger, et al, 1991; Turner, et al; 1992b), and the gene encoding H-protein has been cloned (Macherel, et al, 1992). Moreover, the amino acid sequence of P- and L-subunits of pea leaf has been investigated by Turner et al (1992a) and Bourguignon et al (1992).

On the other hand, serine hydroxymethyltransferase catalyzes a reversible reaction (equation 2). This reversibility accounts for the interconvertibility of glycine and serine in living cells (Schirch, 1984). Higher plants, as well as mammals, contain mitochondrial and cytosolic isoenzymes of SHMT (Cossins, 1987). The higher plant mitochondrial enzyme is responsible for serine synthesis during photorespiration (Tolbert, 1980).

C_1 substituted folates can also be generated from formate via an ATP-dependent

reaction catalyzed by formyl-tetrahydrofolate synthetase, E.C. 6.3.4.3 (equation 3). This

$$\text{Formate} + \text{ATP} + H_4\text{PteGlu} = 10\text{-}HCOH_4\text{PteGlu} + \text{ADP} + \text{Pi} \quad (3)$$

enzyme is part of a multifunctional complex termed C_1-tetrahydrofolate synthase (C_1-THF synthase) in mammalian tissues (Tan, et al, 1977; Schirch, 1978) and in yeast (Appling and Rabinowitz, 1985). Recent studies of spinach leaves (Nour and Rabinowitz, 1992) and pea seedlings (Kirk et al, 1993) show that these tissues contain a monofunctional formyltetrahydrofolate synthetase protein.

1-1-2. The Interconversion of C_1 Units

In order to involve folates as donors of C_1 units, cells must be able to convert C_1 substituted folates to different oxidation states in response to various metabolic demands (Dev and Harvey, 1978). Regulating the flow of the C_1 units through C_1 substituted folate derivatives allows cells to divert the limited supply of these coenzymes to satisfy the most immediate requirements of the cell (Appling and Rabinowitz, 1985). The key enzymes in this intercon-version of folates are 10-formyltetrahydrofolate synthetase, 5,10-methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9) and 5,10-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5). These three enzymes catalyze the reversible reactions as shown in equations 3, 4, and 5, respectively.

$$10\text{-}HCOH_4\text{PteGlu} + H^+ = 5,10\text{-}CH{\equiv}H_4\text{PteGlu} + H_2O \quad (4)$$

$$5,10\text{-}CH{\equiv}H_4\text{PteGlu}_n + \text{NADPH} = 5,10\text{-}CH_2{=}H_4\text{PteGlu}_n + \text{NADP}^+ \quad (5)$$

These three enzyme activities are associated with a single polypeptide in animal tissues (Tan et al, 1977; Paukert et al, 1976; Schirch, 1978; Wasserman et al, 1983). The loc. ization of the gene encoding the human trifunctional enzyme was studied by using male and female subjects (Rozen et al, 1989). In *Saccharomyces cerevisiae*, both the cytoplasmic and mitochondrial isozymes are trifunctional and are encoded by the genes ADE3 and MIS1, respectively (Appling and Rabinowitz, 1985; Shannon and Rabinowitz, 1988). These trifunctional enzymes both possess NADP-dependent methylenetetrahydrofolate dehydrogenase while recent studies of yeast revealed a monofunctional, cytoplasmic NAD-dependent methylenetetrahydrofolate dehydrogenase (Barlowe and Appling, 1990; West et al, 1993). In prokaryotes, various forms of these three enzymes have been identified from different organisms (Ljungdahl et al, 1980; D'Ari and Rabinowitz, 1991; West et al, 1993). Studies of the structural organization of these three enzymes in higher plants was attempted only recently (Nour and Rabinowitz, 1988). The synthetase protein of spinach leaves (Nour and Rabinowitz, 1991) and pea seedlings (Kirk et al, 1993) does not copurified with methylenetetrahydrofolate dehydrogenase and methenyltetrahydrofolate cyclohydrolase activities. The amino acid sequence of the spinach synthetase was compared with the synthetase domains of four trifunctional eukaryotic C_1-THF synthases and two monofunc-tional prokaryotic synthetases (Nour and Rabinowitz, 1992). This plant enzyme had greater homology with the mammalian proteins than those of yeast or bacterial origin.

1-1-3. The Utilization of C_1 Units

The C_1 units at the N^5 and/or N^{10} positions of C_1 substituted folates can be directed into three cytoplasmic one-carbon cycles of methionine, *de novo* purine, and thymidylate synthesis (Shane, 1989). Moreover, the initiation of polypeptide synthesis in prokaryotic organisms and in the mitochondria and chloroplasts of eukaryotes also

involves the C_1 substituted, folate-dependent reactions.

1-1-3-1. The Methionine Cycle

The methionine cycle is recognized as a major route for C_1 unit utilization. This cycle involves the reduction of 5,10-CH_2=H_4PteGlu$_n$ to 5-CH_3-H_4PteGlu$_n$ followed by transfer of the methyl group to homocysteine to form methionine and to regenerate H_4PteGlu$_n$ (Banerjee and Matthews, 1990). 5,10-CH_2=H_4PteGlu$_n$ reduction is catalyzed by methylenetetrahydro-folate reductase (EC 1.1.1.68) as shown in equation 6.

$$5,10\text{-}CH_2\text{=}H_4PteGlu_n + NADPH \rightleftharpoons 5\text{-}CH_3\text{-}H_4PteGlu_n + NADP \quad (6)$$

The reaction is irreversible under *in vitro* and *in vivo* conditions, and is the committed step in methionine synthesis (Shane, 1989). The enzyme, methylenetetrahydro-folate reductase, has been reported from a variety of species (Zhou et al, 1990; Wohlfrath et al, 1990).

Methionine synthase (EC 2.1.1.13) catalyzes the transfer of the methyl group from 5-CH_3-H_4PteGlu$_n$ to homocysteine as shown in equation 7.

$$5\text{-}CH_3\text{-}H_4PteGlu_n + \text{homocysteine} \rightleftharpoons H_4PteGlu_n + \text{methionine} \quad (7)$$

The methionine synthase reaction is the sole reaction whereby the methyl group of 5-CH_3-H_4PteGlu$_n$ can be metabolized in mammalian cells (Shane, 1989). On the other hand, this reaction represents the terminal step of methionine biosynthesis in higher plants and prokaryotes that synthesize methionine *de novo* (Banerjee and Matthews, 1990). The mammalian synthase contains tightly bound cobalamin and the reaction proceeds via a methylcobalamin intermediate. Higher plants and fungi have a different

type of methionine synthase which is cobalamin-independent (Cossins, 1987). *Escherichia coli* cells express both types of methionine synthase (Banerjee and Matthews, 1990; Banerjee et al, 1990).

1-1-3-2. The Thymidylate Cycle

Folate is also required for the synthesis of thymidylate. This process involves a cyclic sequence catalyzed by thymidylate synthase (TS) EC 2.1.1.45, dihydrofolate reductase (DHFR) EC 1.5.1.3, and SHMT. Since thymidylate (as its triphosphate derivative) is considered to be a limiting component in DNA synthesis, TS and DHFR have received extensive attention as targets for cancer chemotherapy (Huennekens et al, 1987; Fleming and Schilsky, 1992; Rosowsky et al, 1992). TS catalyzes the transfer of formaldehyde from folate to the 5-position of deoxyuridylate as shown in equation 8. The pyrazine ring of $H_4PteGlu_n$

$$5,10\text{-}CH_2{=}H_4PteGlu_n + dUMP{=}H_2PteGlu_n + dTMP \qquad (8)$$

provides the reducing component for the reduction of the transferred one-carbon moiety to the level of methanol, and $H_4PteGlu_n$ is oxidized to $H_2PteGlu_n$ in the process (Shane, 1989). $H_2PteGlu_n$ is inactive in C_1 transfer reactions and is reduced to $H_4PteGlu_n$ by DHFR as shown in equation 9. The

$$H_2PteGlu_n + NADPH{=}H_4PteGlu_n + NADP \qquad (9)$$

$H_4PteGlu_n$ can then be reconverted to $5,10\text{-}CH_2{=}H_4PteGlu_n$ by SHMT (equation 2) and complete the cycle.

The levels of TS in mitotically inactive tissues are practically nonexistent (Maley and Maley, 1990) but the expression of the synthase, as well as DHFR, is highest

during the S phase of the cell cycle (Shane, 1989). TS and DHFR exist as distinct and readily separable proteins in bacteriophage, mammalian viruses, yeast, and vertebrates (Ivanetich and Santi, 1989). In protozoa, TS and DHFR exist on the same polypeptide (Ivanetich and Santi, 1990). A fair amount of attention has been paid to the TS of *Pneumocystis carinii* in recent years. In developed nations, *P. carinii* pneumonia (PCP) is the most common lethal infection of patients with acquired immuno-deficiency syndrome, or AIDS (Ivanetich and Santi, 1989; Amsden, et al, 1992). Since TS is the sole *de novo* source of dTMP synthesis, and thymine depletion is cytotoxic to these organisms, TS is an attractive target for the development of chemotherapeutic agents. The TS gene of *P. carinii* has recently been isolated and expressed in *E. coli* (Edman et al, 1989).

DHFR is now one of the most well-characterized enzymes. This protein has been invastigated by crystallographic (McTigue et al, 1992) and NMR studies (Feeney, 1992; Gerothanassis, 1992). In addition, there is considerable information regarding its catalytic properties and genetic expression (Freisheim and Matthews, 1984). Since the 1940's, DHFR has been a target of 4-amino analogs of folate in clinical treament of a variety of diseases such as childhood acute leukemia (Fleming and Schilsky, 1992), bacterial and protozoan infections (Feeney, 1990; Ivanetich and Santi, 1990), and PCP accompanied with AIDS (Amsden et al, 1992). Even today, the classical chemotherapeutic agent methotrexate (MTX) remains an important folate antagonist used in cancer chemotherapy. The historical perspectives of research on this key enzyme of C_1 metabolism have been reviewed recently in the 1988 Nobel Lecture in Physiology or Medicine by Dr. G. H. Hitchings (1989). The major role of DHFR in mammalian cells is the reduction of $H_2PteGlu_n$ formed during thymidylate synthesis. Since replicating cells must synthesize thymidylate either from exogenously supplied thymidine or by the TS reaction, DHFR must reduce $H_2PteGlu_n$ as rapidly as it is formed by TS to avoid depletion of intracellular $H_4PteGlu_n$. This is especially

important to rapid dividing tumor cells. On the other hand, this enzyme from prokaryotes and higher plant tissues also actively participates in the *de novo* biosynthesis of folic acid. The latter role of DHFR will be discussed in the next section. The reader's attention is directed to excellent reviews by Blakley (1984) for more information on the general properties of this enzyme, and by Hamlin and Ma (1990) for more imformation on studies of the DHFR gene.

1-1-3-3. The Purine Cycle

The De novo biosynthesis of purines also requires folate derivatives. The C-8 and C-2 of the purine ring are derived from 10-formyl-$H_4PteGlu_n$ in reactions catalyzed by glycinamide ribonucleotide (GAR) transformylase, EC 2.1.2.2 and 5-amino-4-imidazolecarboxamide ribonucleotide (AICAR) transformylase, EC 2.1.2.3. GAR transformylase catalyzes the transfer of the formyl group of 10-formyltetrahydrofolate to the amino group of GAR to form formyl-glycinamide ribonucleotide (FGAR) as shown in equation 10. The carbon of this formyl group is then incorporated in C-8 position.

$$10\text{-HCO-}H_4PteGlu_n + GAR = HCO\text{-}GAR + H_4PteGlu_n \qquad (10)$$

AICAR transformylase catalyzes the transfer of formyl group from 10-HCO-$H_4PteGlu_n$ to AICAR (equation 11), and the carbon

$$10\text{-HCO-}H_4PteGlu_n + AICAR = HCO\text{-}AICAR + H_4PteGlu_n \qquad (11)$$

of this formyl group is then incorporated into C-2 position of the purine ring. The protein structure of GAR transformylase has been reported (Daubner et al, 1985; Henikoff et al, 1986). The crystalline and atomic structures of GAR transformylase

have also been investigated (Smith and Daum, 1987; Chen et al, 1992). The cDNAs encoding this enzyme has been cloned from human and avian tissues (Aimi et al, 1990). GAR transformylase does not appear to have been characterized from a higher plant source (Cossins, 1987). However, AICAR transformylase has been isolated from various sources including higher plant tissues (Iwai, 1972).

1-1-3-4. The Initiation of Protein Synthesis

The initiation of polypeptide synthesis in prokaryotic organisms and in the mitochondria and chloroplasts of eukaryotes, requires formylmethionyl tRNA (Staben and Rabinowitz, 1984). Protein synthesis and C_1 metabolism are closely linked as this initiator receives formyl groups exclusively from 10-HCO-H_4PteGlu. The generation of formyl-methionyl tRNA (fMet-tRNA) is via a folate-dependent reaction catalyzed by methionyl-tRNA transformylase (EC 2.1.2.9) as shown in equation 12. The enzyme has been

$$\text{10-HCO-}H_4\text{PteGlu} + \text{Met-tRNA} = \text{fMet-tRNA} + H_4\text{PteGlu} \qquad (12)$$

purified from a variety of sources (Staben and Rabinowitz, 1984), and the *E. coli* gene encoding this enzyme has been characterized (Lee et al, 1991; Varshney and RajBhandary, 1992).

1-2 Folylpolyglutamates: Biosynthesis and Degradation

The natural occurring forms of folate in plants are tetrahydropteroyl-gamma-polyglutamates containing 3-8 glutamic acid residues (Imeson, et al, 1990; Zheng, et al, 1992) as shown in Fig. 2. Originally, these polyglutamates were believed to be storage forms of the coenzyme (Schirch and Strong, 1989; Ward and Nixon, 1990). However, studies of purified enzymes have clearly shown that the polyglutamate forms

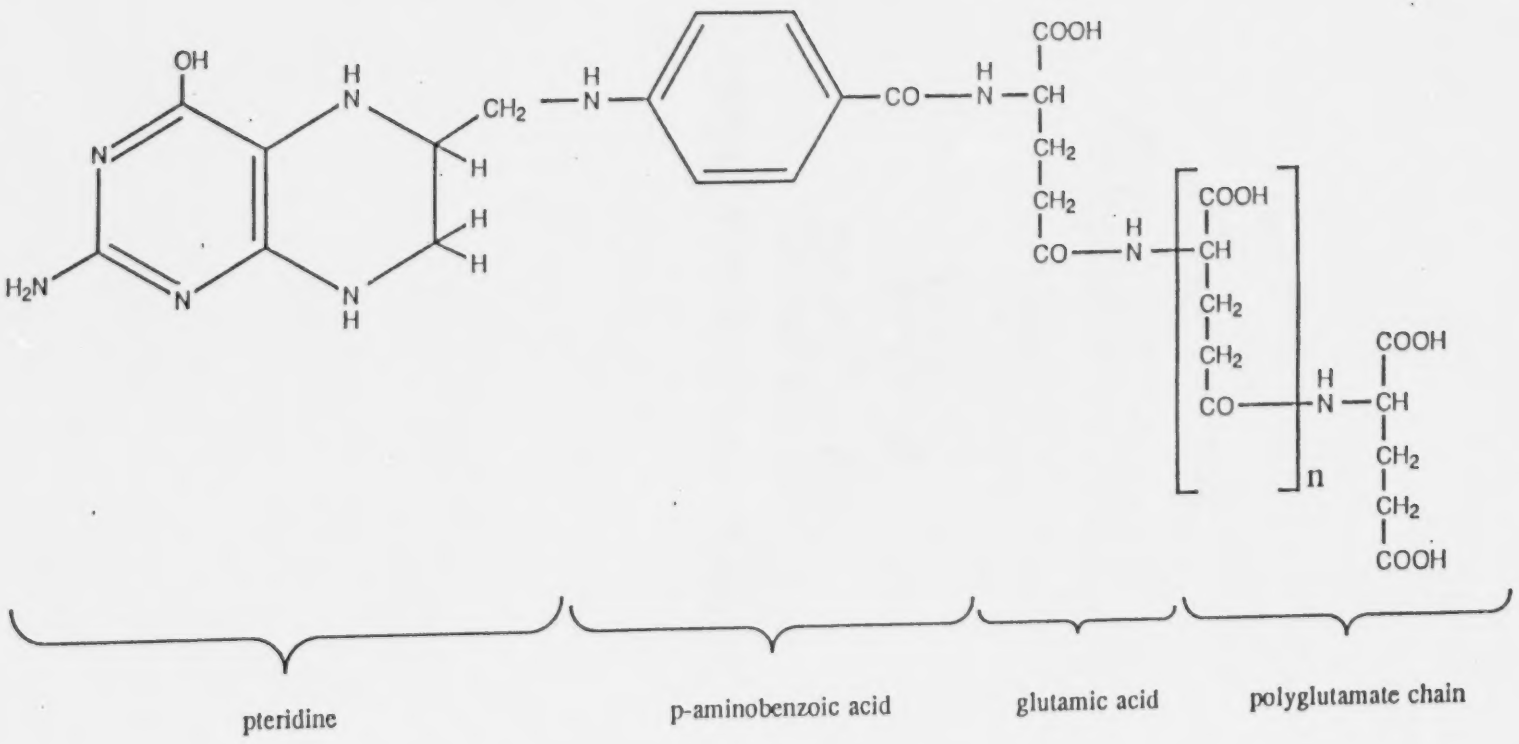


Fig 2. Structure of Folylpolyglutamate. Natural occurring forms of folates are tetrahydropteroyl-gamma-polyglutamates containing 3-8 glutamic acid residues.

are the true physiological substrates and regulators of C_1 metabolism (Cossins, 1980; 1984; 1987; Shane, 1989). More than five decades of research, since the discovery of folic acid, have shown that the polyglutamate tail of folate is very important in C_1 metabolism. The subjects of folylpolyglutamate biosynthesis and degradation have attracted much attention recently (McGuire and Coward, 1984; Kalman et al, 1986; Whitehead, et al, 1987; Whitehead, et al, 1988; Schirch, and Strong, 1989; Barredo and Moran, 1992). It is now clear that most enzymes of C_1 metabolism prefer long-chain folates as substrates (Ross et al, 1984; Shane, 1989). Moreover, the polyglutamate tail appears to play an important role in folate homeostasis and in the cytotoxic efficacy of antifolates (Shane et al, 1990). The polyglutamation of folate derivatives and the factors that regulate the chain-length of these coenzymes would be expected to play a major role in the regulation of folate homeostasis and the regulation of C_1 metabolism. Two enzymes can directly regulate this process. Folylpolyglutamate synthetase (FPGS), EC 6.3.2.17, which catalyzes the synthesis of folylpolyglutamates, and gamma-glutamyl hydrolase (GGH), EC 3.4.22.12, a peptidase that hydrolyzes the folate polypeptide chain. These two enzymes have been the subject of recent studies (Galivan et al, 1987; Pristupa et al, 1991; Garrow et al, 1992; Cody et al, 1992), and represent a new area for cancer chemotherapeutic studies (Johnson et al, 1988; Licato et al, 1990; Kalman, 1990; O'Connor et al, 1991; Barredo and Moran, 1992).

Folates are synthesized *de novo* in plants, fungi and bacteria. On the other hand, mammals lack dihydrofolate synthetase (EC 6.3.2.12), a key enzyme in the *de novo* pathway for folate synthesis, and therefore require a dietary source of folate. The *de novo* synthesis of folate has been reviewed in detail by Cossins (1980). As discussed earlier, DHFR plays an important role in this pathway forming H_4PteGlu (Fig 3) which is subjected to further polyglutamation by FPGS.

FPGS catalyzes the ATP-dependent addition of glutamate to H_4PteGlu (equation 13, n=1 to 7). The substrate, H_4PteGlu, can be either from *de novo* synthesis or,

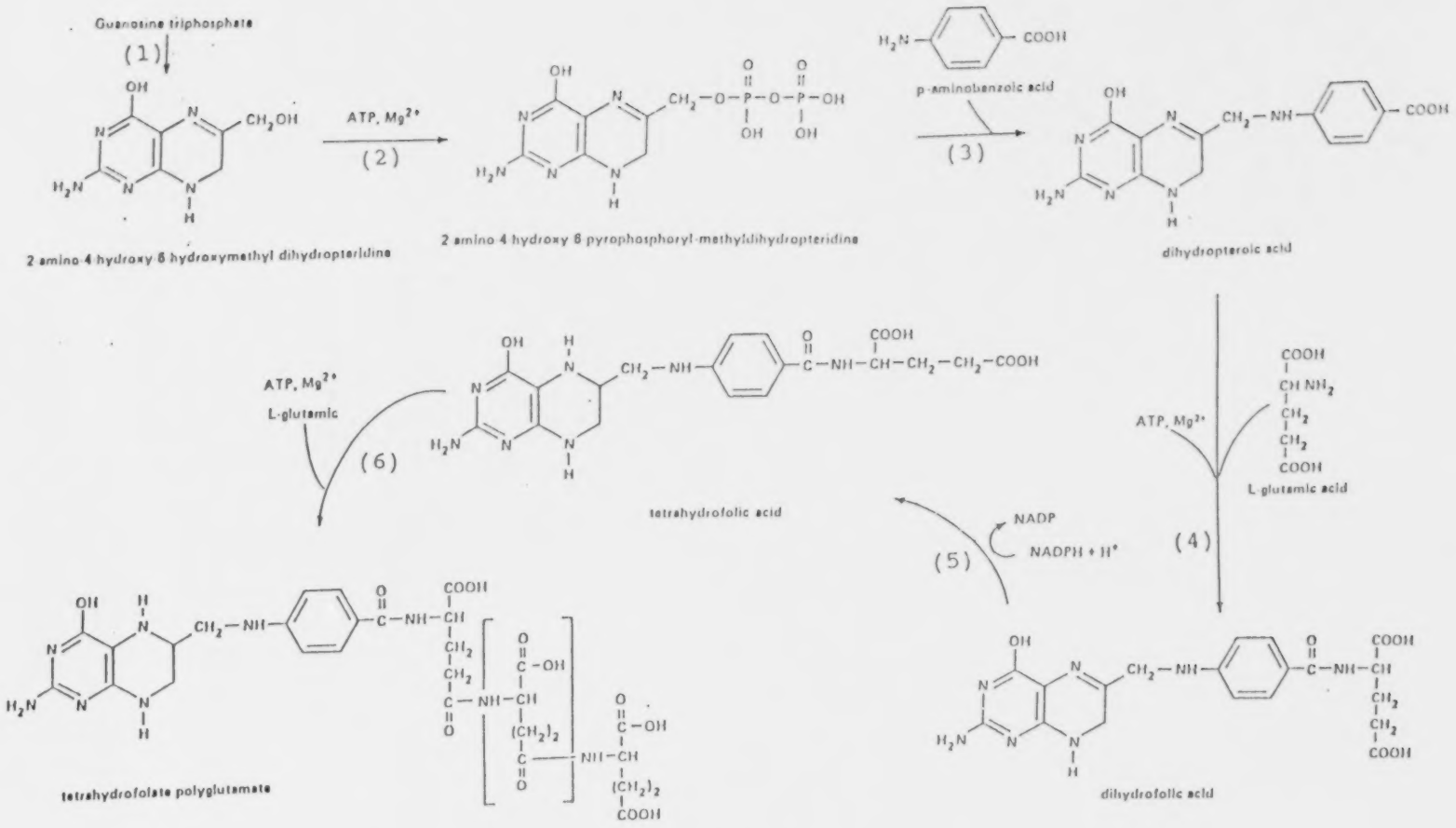


Fig. 3. *De novo* pathway of folate biosynthesis. Enzymes catalyze these reactions are (1) multiple-step process involves several enzymes. (2) hydroxymethyldihydropterin pyrophosphokinase (3) dihydropteroate synthase (4) dihydrofolate synthetase (5) DHFR (6) FPGS.

$$H_4PteGlu_n + Glu + MgATP \rightarrow H_4PteGlu_{n+1} + MgADP + Pi \quad (13)$$

in mammalian tissues, will be derived from the diet. Furthermore, some antifolate drugs including methotrexate (MTX) serve as substrates of FPGS. Plants, mammals and fungi have lower levels of FPGS protein than prokaryotic species (Shane, 1989). FPGS has been reported from pea cotyledons (Chan et al, 1986; Imeson and Cossins, 1991a; Imeson and Cossins, 1991b). Bacterial FPGS proteins differ markedly from those of eukaryotic sources in terms of their substrate specificity and bifunctional nature. (Shane, 1989).

From a nutritional point of view, the polyglutamate nature of dietary folate affects its bioavailability in human cells (Gregory et al, 1991). Thus intestinal absorption of dietary folate requires hydrolysis of pteroylpolyglutamate forms to pteroylmonoglutamate. This led to the discovery of two separate GGH activities in human jejunum (Reisenauer et al, 1977).

Gamma-glutamyl hydrolase, also called by other names such as pteroylpolyglutamate hydrolase and folate conjugase, has a universal distribution throughout the phylogenetic spectrum (Silink et al, 1975). However, hydrolases from various sources have different properties in terms of their catalytic mechanisms. One type of GGH acts as an endopepti-dase, cleaving the innermost gamma-peptide bond of folyl- or antifolylpolyglutamates to yield monoglutamate products. Another type of GGH acts as an exopeptidase and liberates the hydrolytic products in a progressive stepwise manner. This latter group of enzymes is sometimes referred to as gamma-glutamyl carboxypeptidase (Hoffbrand and Peters, 1969; Chandler et al, 1986). Based on these modes of action, the hydrolysis of $PteGlu_n$ can be illustrated as in equation 14 and 15.

$$PteGlu_n + H_2O \rightleftharpoons PteGlu + Glu_{n-1} \quad (14)$$

$$PteGlu_n + H_2O \rightleftharpoons PteGlu_{n-1} + Glu \qquad (15)$$

GGH was found in both intracellular and plasmal membrane fractions in mammalian intestinal tissues (Wang et al, 1985). Although the significance of the membrane-bound enzyme in dietary folate uptake has been widely recognized (Halsted, 1991), the function of the soluble GGH remains unclear.

In plant tissues, gamma-glutamyl hydrolase has been reported for cabbage (Tamura et al, 1972), spinach and pea seedling (Iwai, 1957). In crude extracts of cabbage, two separate GGH activities were detected by microbiological assay. These hydrolase activities exhibited acidic or basic pH optimum. Later studies of pea seedlings (Lin et al,1993), showed that the enzyme has different properties compared to some mammalian GGH proteins (Wang et al, 1985). Despite these reports, the role of plant GGH activities in one-carbon metabolim remains obscure.

1-3 GGH Activity and Cross-Membrane Transport of Folate

GGH activity and the intestinal absorption of dietary folates in healthy and diseased subjects has been studied extensively by clinical nutritionists. Absence or low GGH activity causes folate deficiency which in turn causes diarrhea and malabsorption of water-soluble nutrients (Halsted, 1975). Moreover, chronic alcoholism and cigarette smoking may also cause folate deficiency by inhibiting membrane GGH activity (Naughton, 1989; Piyathilake et al, 1992).

A two-step process for dietary folate uptake has been proposed based on the observation that hydrolysis of folate occurs on the surface of the jejunum and is a prerequisite for folate absorption (Halsted, 1991). This process involves the hydrolysis of glutamate side chain by membrane-bound GGH in brush border followed by the cross-membrane transport of the monoglutamate derivative into the enterocyte. Although the mechanism of the second step in this process is controversial, the

requirement for brush border GGH before folates cross the membrane is well recognized.

Jejunal brush border GGH is an exopeptidase with neutrual pH optimum (Reisenauer and Halsted, 1981). This enzyme activity is activated by zinc and not inhibited by pHMB, a sulfhydryl inhibitor (Wang et al, 1985; Chandler et al, 1986; Gregory et al, 1987). In contrast, intracellular GGH from various mammalian sources displays endopeptidase activity and has an acidic pH optimum. This soluble enzyme is sensitive to pHMB (Reisenauer et al, 1977).

GGH activity also has great pharmacological value and is a target for cancer chemotherapeutic drug design (Kalman, 1990). The rational design is based on the evidence that GGH is involved in folyl- or antifolylpolyglutamate turnover (Whitehead et al, 1987; 1988; 1990; Kalman, 1990) and therefore affects folate transport across the cellular membrane (Galivan, 1979; Whitehead et al, 1990; Barrueco et al, 1992). For example, the folate analog MTX is converted to polyglutamate derivatives by normal and tumor cells (Galivan, 1979; Whitehead et al, 1990). The polyglutamate forms of MTX (MTXPG) have longer half-lives within the cell and therefore their cytotoxicity is enhanced. On the other hand, GGH hydrolyzes MTXPG and allows the hydrolytic products to efflux from the cell. This role of GGH was confirmed by treating cultured leukemic cells with the GGH inhibitor 2-mercaptomethylglutaric acid (Whitehead et al, 1990).

Despite all these detailed studies of human and animal tissues, GGH in plants has received little attention. Earlier studies of cabbage (Tamura et al, 1972) and soybean leaf (Iwai, 1957) were limited as relatively crude extracts were investigated. However, higher plants have active pathways of one-carbon metabolism and therefore provide the basis for research on the synthesis and degradation of folylpolyglutamates. Moreover, vegetables are the major sources of folate for human beings and GGH is the enzyme that greatly affects the dietary uptake of this important vitamin. In present study, GGH

has been isolated and characterized from pea cotyledons, a tissue that synthesizes folylpolyglutamates during germination (Cossins, 1980). The properties of this plant enzyme have also been compared with those of GGH from other biological sources. The possible role of this enzyme in the folate metabolism of this plant tissue is also considered. Part of this thesis research has been published (Lin et al, 1993).

MATERIALS AND METHODS

2-1 Chemicals

$PteGlu_n$ (n=3,5), p-$ABAGlu_n$ (n=1-6) and gamma-glutamyl di- and tripeptides were obtained from Dr. B. Schircks Laboratories, Jona, Switzerland. [^{14}C]-$PteGlu_3$, labelled in the terminal glutamate moiety, and supplied by Dr. Carlos Krumdieck, Department of Biochemistry, University of Alabama, Birmingham, was diluted with carrier $PteGlu_3$ to give 0.033 uC/0.1 umol/ml of solution. Yeast carboxy-peptidase Y, heparin agarose and molecular weight marker proteins were from Sigma.

2-2 Plant Material

Seeds of pea (*Pisum sativum* L. cv. Homesteader) were from a local commercial source and seedlings were produced under controlled growth conditions (Imeson et al, 1990). Spinach leaves were obtained from local market sources.

2-3 Assay of GGH Activity

The enzymatic hydrolysis of [^{14}C]-$PteGlu_3$ and $PteGlu_5$ was examined by the method of Krumdieck and Baugh (1970). Reaction systems (0.8 ml) containing 60 umol acetate buffer (pH 6), 10 nmol folate substrate and ca. 0.1 ug to 0.1 mg of plant protein were incubated at 37^{o}C for 30 min. When release of [^{14}C]-diglutamyl peptide was followed, the reaction was terminated with 0.5 ml 12.5% TCA and excess substrate was removed using activated charcoal (Krumdieck and Baugh,1970). After adding 1.25 ml of 0.65% activated charcoal (in 0.1 M acetic acid), the reaction mixture was incubated at room temperature for 15 min with intermittently shaking to allow the excess substrate or folate products to bind to the charcoal. The nonfolate products were then recovered by passage of the suspension through a syringe filter (0.45 um). 1 ml of the filtrate was then counted in the scintillation counter.

2-4 HPLC Analysis of Folate Products

When folate products of the GGH reaction were examined, the reaction was terminated by adding 3 ml of 30 mM 2-mercaptoethanol and boiling for 3 min. To separate the folate and nonfolate products, 2 ml of the mixture was applied to a Whatman DE-52 cellulose column (0.7 X 4 cm Bio-Rad Econo-columns, Bio-Rad Laboratories) equilibrated with 10 mM Tris-HCl buffer (pH 7.5) containing 80 mM NaCl. (Bognar et al, 1985). Collection of 4 ml fractions was started while applying the sample to the column. The columns were then washed with 30 ml of the equilibriating buffer. Folate products were eluted with 8 ml of 0.1 N HCl. Aliquots of each fraction were then counted in the scintillation counter.

Folate products in fractions of the second peak (Fig. 4) were examined by HPLC analysis. In this regard, these peak fractions were adjusted to pH 0.5 with 5 N HCl and stored overnight at $4^{o}C$. Then 0.05 of sample volume of zinc suspension (1 g of Zn metal suspended in 4 ml of 0.5% gelatin) was added to cleave the folates to p-$ABAGlu_n$ (Shane, 1986). This mixture was allowed to react for 10 min with stirring. Particulate Zn was then removed by centrifugation. The supernatant containing p-$ABAGlu_n$, was mixed with 0.3 ml of 0.5% $NaNO_2$ to generate the diazonium salts. After 2-3 min, 0.3 ml of 2.5% ammonium sulfamate was added with mixing to destroy excess HNO_2. After a further 2-3 min, 0.5 ml of 0.1% naphthylethylenediamine was added and the solution was left for at least 30 min at room temperature or overnight at $4^{o}C$ to ensure complete conversion of the diazonium salts to the purple-colored azo dye derivatives. The azo dye solution was applied to a 0.7 x 4 cm Bio Gel P2 (200-400 mesh) column (Bio-Rad Econo column) equilibrated with 0.1 N HCl. The column was then washed with 6 x 1 ml 0.1 N HCl to remove pterin cleavage products, any incomplete folate cleavage products, and nonspecific UV-absorbing material from the tissue extracts. The azo dyes were eluted with 1 ml of 0.1 M potassium phosphate

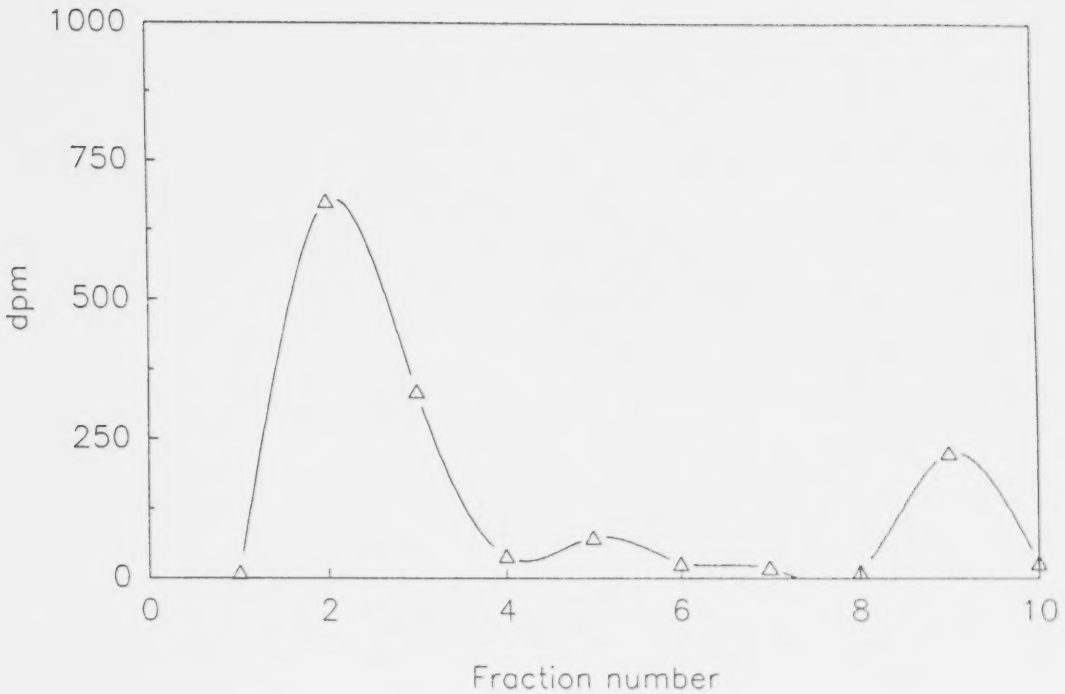


Fig. 4. Separation of folate and non-folate products on DE-52 cellulose column.

The GGH reaction was terminated by adding 3 ml of 30 mM 2-mercaptoethanol and boiling for 3 min. 2 ml of this mixture was applied to a Whatman DE-52 cellulose column (0.7 x 4 cm) pre-equilibrated with Tris-Cl buffer (see Materials and Methods). The column was washed with 8 ml of 0.1 N HCl after collection of fraction 7. The non-folate product was the first eluted material detected by scintillation counter while the folate product formed the second peak at fraction 9. Results are expressed as dpm/ml.

buffer (pH 7.0) and water (5 x 1 ml) as described by Shane (1986).

p-ABAGlu$_n$ were then regenerated from the azo dyes by zinc reduction (Shane, 1986). The solution was filtered to remove zinc and then dried in rotary evaporator at 45^{o}C. The residue was resuspended in 1.5 ml of H_2O and adjusted to pH 6.5-7 by careful addition of 2 N NaOH. The white precipitate of $Zn(OH)_2$ was removed by centrifugation. The supernatant was then filtered through a microfilter (pore size 0.45 mm) prior to HPLC analysis.

Individual p-aminobenzoylpolyglutamates of different chain lengths were separated by HPLC using a 250 x 4.6 mm column of strong anionic exchanger (Partisil 10 SAX, Whatman) and Varian chromatographic system (Shane, 1986). In this way, the folate products of GGH assay were characterized and the hydrolysis mechanism of GGH (exo- or endopeptidase) was examined. The chromatographic program was run at 35^{o}C with the flow rate of 1 ml/min and 200 ul of sample were injected. The column was then eluted with 25 mM ammonium phosphate buffer (pH 6.5) for 10 min, followed by a buffer gradient from 25 mM to 275 mM over 30 min, and from 275 to 500 mM over 45 min. The chain lengths of eluted samples were determined by comparing the retention times with those of p-ABAGlu$_n$ standards.

Labelled reaction products, not absorbed by DE-52 cellulose, were characterized by ion exchange chromatography using Dowex (see Fig. 22 of the Results). The identity of diglutamyl peptide was confirmed by HPLC using a procedure described by Zheng et al (1992).

2-5 GGH Extraction and Purification

Pea cotyledons (50 g) were ground in 100 ml of 0.1 M Na phosphate buffer (pH 6) containing 20% v/v glycerol and 50 mM 2-mercaptoethanol (Buffer A). After filtering through cheese cloth, the homogenate was centrifuged (10,000g for 10 min) to give step 1 protein. Step 2 protein was obtained by removing nucleic acids using

streptomycin sulphate (Zheng, et al, 1992). Protein was fractionated with $(NH_4)_2SO_4$. Step 3 protein precipitating in the 40-70% range of saturation was dissolved in 10 ml of Buffer A and desalted by passage through Bio-Gel P6 (Bio-Rad) column. The extract was then applied to a 2.6 X 20 cm column of DEAE-Sephacel (Pharmacia) previously equilibrated with Buffer A. After washing with 100 ml of this buffer a linear NaCl gradient (0 to 0.6 M in Buffer A) was applied and 6 ml fractions were collected. Fractions containing GGH (step 4 protein) were then applied to a 2.5 X 80 cm column of Sephacryl S 200 (Sigma) previously equilibrated with Buffer A containing 100 mM NaCl. 5 ml fractions were collected at a flow rate of 20 ml/hr. GGH-active fractions (step 5) were then applied to a column (2.5 X 10 cm) of Matrex Green A (Amicon) pre-equilibrated in Buffer A. After washing the column with 100 ml of Buffer A, a linear NaCl gradient (0 to 0.6 M in Buffer A) was applied to elute GGH protein. Fractions of 6 ml were collected and those with GGH were concentrated 20-fold using an Amicon ultra-filtration system (step 6). The extract was finally applied to a 1.5 X 10 cm column of heparin agarose pre-equilibrated in Buffer A. A linear NaCl gradient (0 to 0.6 M) was applied to elute GGH protein. Polyacrylamide gel electrophoresis was carried out in the presence of SDS. Protein was assayed by the Bradford method.

2-6 Isolation of Vacuoles from Pea Cotyledons and Leaves

Vacuoles were isolated from pea cotyledons and leaves of 14-day seedlings. The isolation of vacuoles from cotyledons employed the method of Darwen and John (1989) with minor modification. 20 g of 1 day cotyledons were finely sliced in 100 ml of sorbitol buffer (containing 0.8 M sorbitol, 50mM Tris-Cl, 5 mM EDTA disodium salt, 0.1 mM PEG-3350, 0.1% dextran sulfate and 2 mM DTT, pH7.6). The extract was filtered through one layer of miracloth to give a crude homogenate. This homogenate was centrifuged at 2,000g for 15 min to give a vacuolar pellet and the resulting supernatant was recentrifuged at 20,000g for 15 min. The sedimented and supernatant

fractions from this latter step were assayed for GGH, ADH and alpha-mannosidase activities. The vacuolar pellet was resuspended in 20 ml of resuspension buffer I (0.8 M betaine, 50 mM Mes pH 5.2, 0.1 mM PEG-3350 and 20 mM EGTA) and filtered through a single layer of miracloth. Vacuoles in the filtrate were allow to sediment at room temperature for 1 hour and the supernatant was carefully removed with a Pasteur pipette. The sedimented vacuoles were washed in 20 ml of resuspension buffer I and centrifuged at 2,000 g for 10 min. This washing procedure was then repeated. The pellet was finally resuspended in 2 ml of resuspension buffer II (0.8 M betaine, 20 mM Tris-Mes pH 5.2, and 1 mM DTT). Assay of GGH, alpha-mannosidase and alcohol dehydrogenase (ADH) activities were carried out at each step of this procedure.

Vacuoles were also isolated from green leaves of pea seedlings using a protocol provided by Dr. Michel Neuburger. This protocol involved the prior isolation and purification of intact protoplasts followed by lysis to liberate intact vacuoles. Leaves (10 g), harvested from 2-week pea seedlings were cut into small pieces (*ca.* 1 x 1.5 mm) using a razor blade. These pieces of tissue were then placed in a petri dish and incubated at 25^{o}C for 60 min in plasmolysis medium P_1 (0.5 M sorbitol, 1 mM $CaCl_2$, 10 mM MES, pH 5.5). The medium was removed using a Pasteur pipette. 100 ml of digestion medium P_2 (2% of Cellulase, Calbiochem; 0.5% of Macerozyme R-10, Yakult Biochemicals Co. Ltd., Nishinomiya, Japan; and 0.2% of Pectolyase, Sigma) was added to the tissues and the mixture was incubated at 25^{o}C for about 3 hours with slow agitation. When large amounts of intact protoplasts had been released, as judged by light microscopy, the mixture was filtered through nylon cloth (50 um mesh) and centrifuged at 100 g for 5 min using a swinging bucket rotor. After the supernatant was discarded, the pellet was gently resuspended in protoplast medium P_3 (0.5 M sorbitol, 1 mM $CaCl_2$, 10 mM MOPS, pH 7.0) and again centrifuged at 100 g. The pellet of this low speed centrifugation step was retained. For further purification of protoplasts by gradient centrifugation, this pellet was resuspended in gradient medium G_1 (0.5 M

sucrose, 1 mM $CaCl_2$, 10 mM MOPS, pH 7.0). 5 ml of protoplasts suspended in G_1 were layered at the bottom of a centrifuge tube. 2 ml of medium G_2 (0.4 M sucrose, 0.1 M sorbitol, 1 mM $CaCl_2$, 10 mM MOPS, pH 7.0) was carefully layered on top of G_1. Finally, 1 ml of medium G_3 (0.5 M sorbitol, 1 mM $CaCl_2$, 10 mM MOPS, pH 7.0, same as P_3) was layered on top of G_2 (Fig. 5). After centrifuging at 250 g for 5 min, purified protoplasts were recovered from the interface of G_2 and G_3. The protoplasts were diluted to 5 times volume with buffer P_3. Protoplasts were stable up to 6 hours at room temperature in this buffer.

Ficoll gradient centrifugation was employed to isolate intact vacuoles from protoplast suspension. In this procedure, protoplast suspension was centrifuged at 100 g for 5 min and the pellet was resuspended in buffer V_1 (0.5 M sorbitol, 1 mM $CaCl_2$, 0.05% PVP_{25}, 10 mM MOPS, pH 7.0) at about 3-3.5 x 10^7 protoplasts/ml. 0.4 ml of this V_1-protoplast suspension was quickly diluted in 3.6 ml of buffer V_2 (0.1 M KH_2PO_4/K_2HPO_4, pH 8.0, 1 mM DTT, 1 mM $MgCl_2$, 0.05% PVP_{25}) and the mixture was gently swirled for 4 min to release vacuoles from the lysed protoplasts. Aliquots of the suspension (1 ml) were then applied to a 3 layered Ficoll gradient (see Fig 6). The system was centrifuged at 1,000 g at 17°C for 30 min in a Beckman centrifuge (J21, with swinging bucket rotor JS13). The vacuoles were localized at the interface of 1% and 2% Ficoll. Vacuoles were aspirated using a Pasteur pipette and assayed for GGH and alpha-mannosidase activities.

2-7 Isolation of Mitochondria from Pea Cotyledons

Mitochondria were isolated from pea cotyledons using the method described by Johnson-Flanagan and Spencer (1981). 20 g of pea cotyledons were hand ground in 50 ml of grinding buffer (0.5 M mannitol, 5 mM EDTA, 0.5% fatty acid poor BSA, 0.05% cysteine, 0.05 M TES, pH 6.0) for 90 seconds with a mortar and pestle. The extract was filtered through miracloth and the crude homogenate was centrifuged at 700

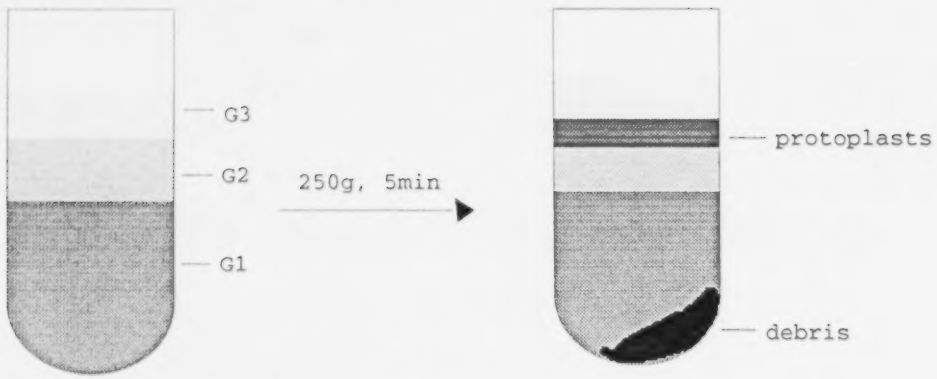


Fig. 5. Purification of protoplasts by gradient centrifugation.

The pellet of low speed centrifugation was resuspended in G_1 (0.5 M sucrose, 1 mM $CaCl_2$, 10 mM MOPS, pH 7) as described in the Materials and Methods. 5 ml of this G_1 suspension were transferred to a centrifuge tube. 2 ml of G_2 (0.4 M sucrose, 0.1 M sorbitol, 1 mM $CaCl_2$, 10 mM MOPS, pH 7) was carefully layered on top of this suspension. Subsequently, 1 ml of G_3 (0.5 M sorbitol, 1 mM $CaCl_2$, 10 mM MOPS, pH 7) was layered on top of G_2 and the tube was spun immediately at 250 g for 5 min in a swinging bucket centrifuge. The intact protoplasts migrated up to the interface between G_2 and G_1, while the debris sank to the bottom of the tube. The protoplasts were transferred to a centrifuge tube and diluted 5 x (volume) with medium G_3, then centrifuged at 100 g for 5 min to obtain the final protoplast pellet.

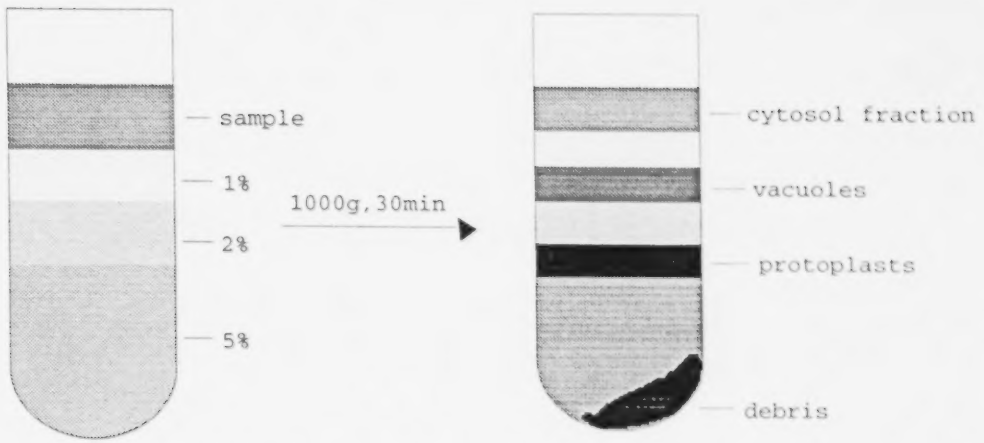


Fig. 6. Isolation of vacuoles from protoplast lysate. 5 ml of 5% Ficoll (w/v, in buffer V_2, 0.1 M KH_2PO_4/K_2HPO_4, pH 8.0, 1mM DTT, 1mM $MgCl_2$, 0.05% PVP_{25}) were layered at the bottom of a centrifuge tube. 2 ml of 2% Ficoll and 1 ml of 1% Ficoll (also in buffer V_2) were subsequently added to the tube to form 3 layers of Ficoll step gradient. The sample of protoplast lysate (1 ml) was layered on the top. This system was centrifuge at 1,000 g for 30 min at 17^{o}C (Beckman J21 centrifuge with JS13 rotor). Vacuoles were recovered from the interface between 1% and 2% Ficoll, while the cytosolic fraction remained on the top and some heavier fractions moved further down.

g for 10 min. The resulting supernatant was then centrifuged at 21,000 g for 15 min to sediment mitochondria. The 21,000 g supernatant was recentrifuged at 100,000 g for 1 hr and assayed for GGH activity. The 21,000 g pellet containing mitochondria was resuspended in wash buffer (0.3 M mannitol, 4 mM $MgCl_2$, 0.05 M TES, pH 6.0) and recentrifuged at 21,000 g for 10 min. The final pellet from this step was resuspended and assayed for GGH, succinic dehydrogenase and ADH activities.

2-8 Preparation of [^{14}C]-Tetrahydrofolatetriglutamate

[^{14}C]-tetrahydrofolatetriglutamate ([^{14}C]-$H_4PteGlu_3$) was prepared by reduction of [^{14}C]-dihydrofolatetriglutamate ([^{14}C]-$H_2PteGlu_3$) catalyzed by dihydrofolate reductase (DHFR). [^{14}C]-$H_2PteGlu_3$ was prepared by dithionite reduction of [^{14}C]-$PteGlu_3$ and purified on a 70 x 1 cm Bio Gel P2 column (Stover and Schirch, 1992). All buffers were prepared using Millipore water and deoxygenated by heating to 80-100^{o}C and then purging with argon until cool. A test tube (100 x 16 mm) containing 1.5 ml 1 N NaOH and 300 mg L-ascorbate was heated to about 80^{o}C and then purged with argon. 2 umole of [^{14}C]-$PteGlu_3$ were then added and the pH was adjusted to 11.2 with NaOH. After adding 70 mg of sodium dithionite followed by a 10-15 min incubation at room temperature, the pH was adjusted to 5.0 using concentrated HCl. After 5 min at room temperature, the test tube was place in an ice bath. Finally, 20 ul of 2-mercaptoethanol were added and the newly formed [^{14}C]-$H_2PteGlu_3$ was purified on Bio Gel P2 column (70 x 1 cm) pre-equilibrated with deoxygenated 20 mM K^+BES buffer (pH 7.5) containing 50 mM 2-mercaptoethanol. [^{14}C]-$H_2PteGlu_3$, the first eluted material, was detected by absorbance at 280 nm. The peak fractions were also verified as [^{14}C]-$H_2PteGlu_3$ by DHFR assay. The active fractions were pooled and concentrated by lyophylization. The lyophylized [^{14}C]-$H_2PteGlu_3$ was then redissolved in 1.5 ml of deoxygenated water containing 20 mM 2-mercaptoethanol and adjusted to pH 7.5. To prepare [^{14}C]-$H_4PteGlu_3$, 1 mg of DHFR

(Biopure, 0.075 units/mg), 1 mg of glucose-6-phosphate dehydrogenase and 9.12 mg of glucose-6-phosphate (Sigma) were added to a vial containing 0.33 umole of NADPH (Sigma). The redissolved [^{14}C]-H_2PteGlu$_3$ was added and the mixture was incubated at 32^{o}C for 4 hours after bubbling argon through for 2 min and quickly capping the vial. The reaction was stopped by heating to 60^{o}C for 5 min. The concentration of [^{14}C]-H_4PteGlu$_3$ was determined by measuring the absorbance at 298 nm. The molar extinction coefficient for H_4PteGlu$_3$ was taken as 28 x 10^3 M^{-1} cm^{-1} (Huennekens et al, 1963).

RESULTS

3-1 GGH Activity of Pea Cotyledons

Extracts of pea cotyledons were found to contain GGH activity and this was affected by use of different extraction buffers (Table 1). Higher GGH activity was extracted using Na phosphate buffer containing 20% of glycerol and this activity was more stable. Unlike liver GGH (Silink et al, 1975), the activity of pea cotyledons was not enhanced by the addition of Zn ions. In crude extracts, the plant enzyme was unstable at $4^{o}C$, losing more than 50% of the initial activity after 15 days storage. These losses were reduced when the extraction buffer contained glycerol. Therefore, a Na phosphate buffer containing 20% glycerol was used as the extraction buffer in all subsequent work.

Maximal hydrolysis of [^{14}C]-$PteGlu_3$ occurred at pH 6.0 and 50% of this maximum was detected at pH 4.5 and 7.2. Unlike the GGH activities of cabbage, where two distinct pH optima at 5.0 and 8.0 have been reported (Tamura et al, 1972), the pea cotyledon enzyme was active over a wide pH range (Fig 7) with $PteGlu_3$ as substrate. Similar data were obtained in reactions containing $PteGlu_5$ (data not shown).

During seedling growth, the specific activity of GGH in cotyledon extracts increased for the first 5 days (Table 2). GGH activity was also found in the leaves, roots, and etiolated shoot of pea seedlings. On a per gram fresh weight basis, green pea leaves were a richer source of GGH than cotyledons. In the latter tissue, activity remained relatively constant throughout the 7 day period of seedling growth (Table 2). Cotyledons and green leaves were used to determine the subcellular localization of this pea GGH.

The possible compartmentation of GGH activity in pea was examined by differential centrifugation (Table 3). The lysosomal localization of some mammalian GGH proteins (McGuire and Coward, 1984) prompted an examination of the vacuolar fractions of

Table 1. Effects of extraction buffer and storage at 4°C on pea cotyledon GGH activity.

Buffer 10 mM, pH 6.0.	GGH activity (nmole/min/g.f.wt.) after storage at 4° (Days) 0	3	6	15
Na acetate buffer:				
no addition	6.97	4.88	3.65	0.35
+ 20% glycerol	7.35	5.50	2.86	4.33
+ 1 mM Zn acetate	1.84	1.51	1.82	1.28
+ 1 mM Zn acetate and 20% glycerol	2.12	1.38	1.19	2.56
Na phosphate buffer:				
no addition	7.70	6.80	1.93	3.29
+ 20% glycerol	7.59	6.75	4.68	5.96

25 g samples of one day old pea cotyledons were homogenized in the buffers indicated and Step 1 protein (see Materials and Methods) was assayed for GGH activity. Data are averages of two separate determinations.

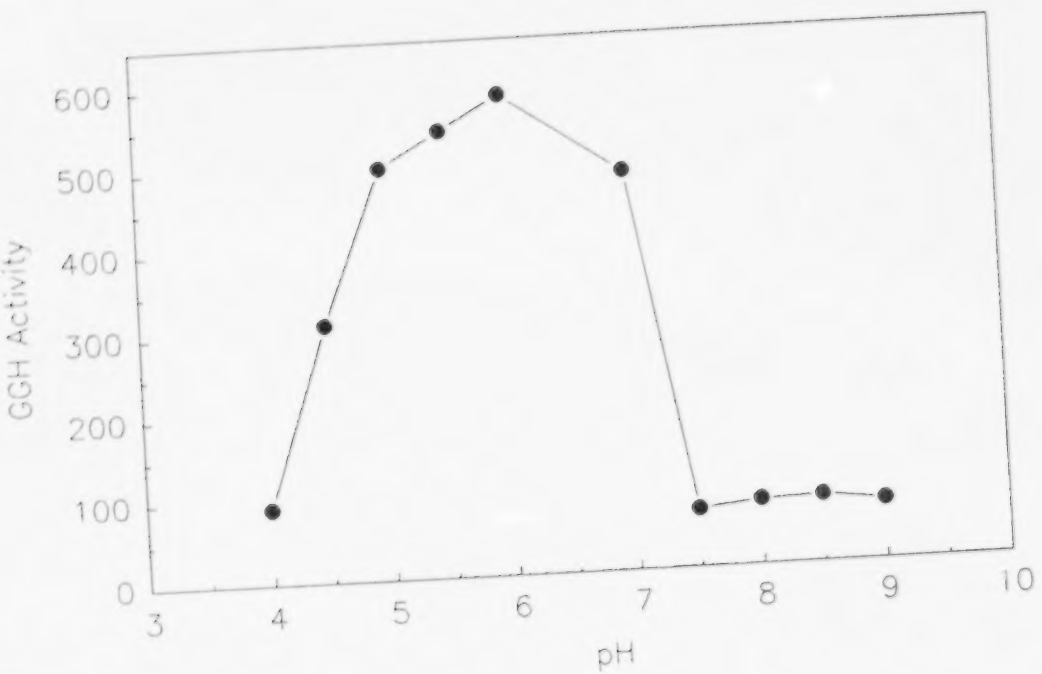


Fig. 7. Effect of assay pH on GGH activity.

0.8 ml assay mixtures, containing 0.6 ml of 0.1 M Na acetate buffer (for pH range of 4-6) or 0.05 M Tris-Cl buffer (for pH range of 7-9), 0.1 ml of concentrated step 6 protein (*ca.* 5 ug), and 0.1 ml of $PteGlu_3$ substrate (10 nmole), were incubated at 37°C for 30 min. The reaction was stopped by adding 0.5 ml of 12.5% TCA. After charcoal treatment (see Materials and Methods), 1 ml of the reaction fluid was counted in the scintillation counter. GGH activities are expressed as dpm/ml. Results are the averages of two separate assays.

Table 2. Changes in GGH activity during pea seedling growth.

Tissue and age	GGH activity* units/mg protein	units/g.fr.wt.	units/cotyledon
Cotyledons:			
1 day	0.66 ± 0.03	11.45 ± 0.79	2.74 ± 0.19
2 day	0.94 ± 0.16	10.76 ± 1.31	2.69 ± 0.33
3 day	1.05 ± 0.53	8.59 ± 3.43	2.03 ± 0.82
4 day	1.36 ± 0.44	12.81 ± 5.13	3.20 ± 1.28
5 day	1.99 ± 0.05	12.75 ± 0.38	3.19 ± 0.10
6 day	1.93 ± 0.34	11.79 ± 1.04	2.95 ± 0.26
7 day	1.57 ± 0.59	12.87 ± 2.07	3.22 ± 0.52
Etiolated Shoots:			
8 day	3.48 ± 1.28	3.91 ± 0.32	
Roots:			
8 day	9.66 ± 3.01	6.91 ± 0.87	
Leaves:			
14 day	1.19 ± 0.19	25.33 ± 4.17	

* Data are the averages (±SD) of four separate extractions. 1 unit= 1 nanomole of $PteGlu_3$ hydrolyzed/min under the defined standard reaction conditions (see Materials and Methods).

Table 3. Intracellular distribution of GGH activity.

Cellular fractions after centrifugation	GGH activity* Total (nmoles/min)	Specific (nmoles/min/mg protein)
1. Vacuolar isolation:		
Crude homogenate	9.58±0.15	1.67±0.47
Washed vacuolar pellet	1.82±0.21	0.06±0.02
20,000g supernatant	8.33±0.32	2.28±0.65
2. Mitochondrial isolation:		
Crude homogenate	104.40±0.67	0.15±0.06
700g pellet	20.46±0.55	0.11±0.02
21,000g pellet (mitoch)	0.43±0.09	0.12±0.05
21,000g supernatant	71.40±0.98	0.20±0.03
100,000g supernatant	67.44±1.09	n.d.

* Data are averages of two separate determinations. n.d. = not determined.

Note: Alpha-mannosidase activity was examined to determine the location of vacuole fractions. Succinate dehydrogenase and alcohol dehydrogenase were assayed to determine the mitochondrial and cytosolic fractions, respectively.

cotyledons and leaves for this enzyme. A vacuolar fraction from cotyledons contained high alpha-mannosidase activity, an established vacuolar marker enzyme (Boller and Kende, 1979), but the associated GGH activity was only a minor part of that detected in the crude homogenate or the final supernatant (Table 3). Vacuoles obtained from green leaves by enzyme digestion also lacked detectable GGH activity (data not shown). Mitochondrial isolations showed only minor GGH activities in contrast to the substantial levels of the enzyme found in the high speed supernatant fraction (Table 3). These data were also confirmed when highly purified pea leaf vacuoles and mitochondria were supplied by Dr. Michel Neuburger, Centre d'Etudes Nucleares, Grenoble, France (data not shown).

3-2 Partial Purification of GGH Protein

Initial attempts to purify GGH from 1 day cotyledons resulted in large losses of activity and final purifications of *ca* 60 fold. However when the same protocol was applied to extracts of 6 day cotyledons (Table 4) GGH activity was highly purified with recoveries of *ca* 10%.

The specific activity of the crude homogenate increased 2-fold following streptomycin sulphate treatment. Subsequent ion exchange chromatography (Fig. 8), of $(NH_4)_2SO_4$-precipitated protein, gave a further 8-fold increase. The resulting step 4 protein was immediately applied to a 2.6 X 80 cm column of Sephacryl S 200 (Fig. 9) and GGH activity was recovered by elution at a flow rate of 20 ml hr^{-1}. Pooled GGH-active fractions after this step had specific activities about 50-fold higher than the initial homogenate (Table 4). Substantial purification occurred when step 5 protein was applied to a column of Matrex Green A (Fig. 10) followed by elution in response to a linear chloride gradient (see Materials and Methods). The GGH-active fractions (step 6 protein) were pooled, concentrated by ultra-filtration and examined by SDS-polyacrylamide electrophoresis. A major protein band, migrating with a M_r of *ca* 52

Table 4. Purification of GGH activity from 6 day old pea cotyledons.

Fractionation step	Volume (ml)	GGH (units*/ml)	Protein (ug/ml)	Spec. Act. (units/mg)	Purification (x-fold)	Yield (%)
1. Crude extract	53	2.44	9690.00	0.25	1.00	100
2. Streptomycin SO_4	50	2.67	5590.00	0.48	1.89	100
3. 40-70% $(NH_4)_2SO_4$**	6.8	2.98	12900.00	0.23	--	--
4. DEAE-Sephacel	28	2.55	638.00	3.99	15.96	55.1
5. Sephacryl S-200	25	1.06	84.00	12.62	50.48	20.5
6. Matrex Green A***	50	0.30	0.03	11337.53	45350.12	11.5

* 1 unit = 1 nmole of $PteGlu_3$ hydrolyzed/min.

** GGH activity assayed after desalting $(NH_4)_2SO_4$-precipitated protein.

*** Protein was assayed after Matrex Green A fractions were pooled and concentrated.

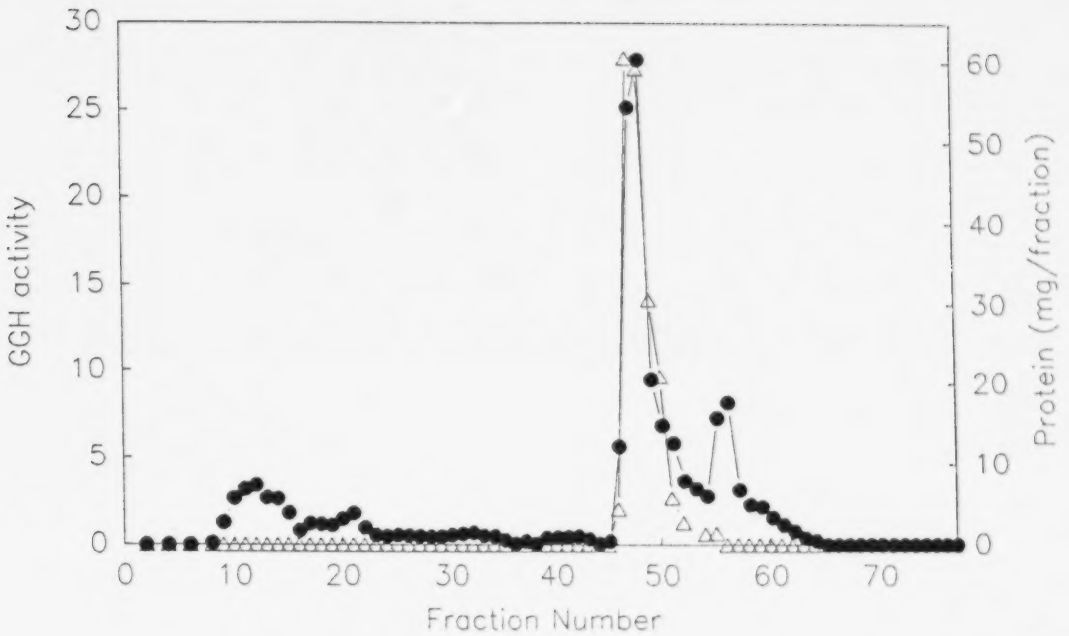


Fig. 8. Chromatography of GGH protein on DEAE-Sephacel. 36 ml of desalted Step 3 protein were applied to a column of DEAE-Sephacel and GGH activity was eluted by applying a gradient of NaCl after collection of fraction 17 (see Materials and Methods). Fractions (6 ml) were assayed for GGH activity (Δ) and protein (●). Enzyme activity is expressed in nmoles $PteGlu_3$ hydrolyzed/min/fraction.

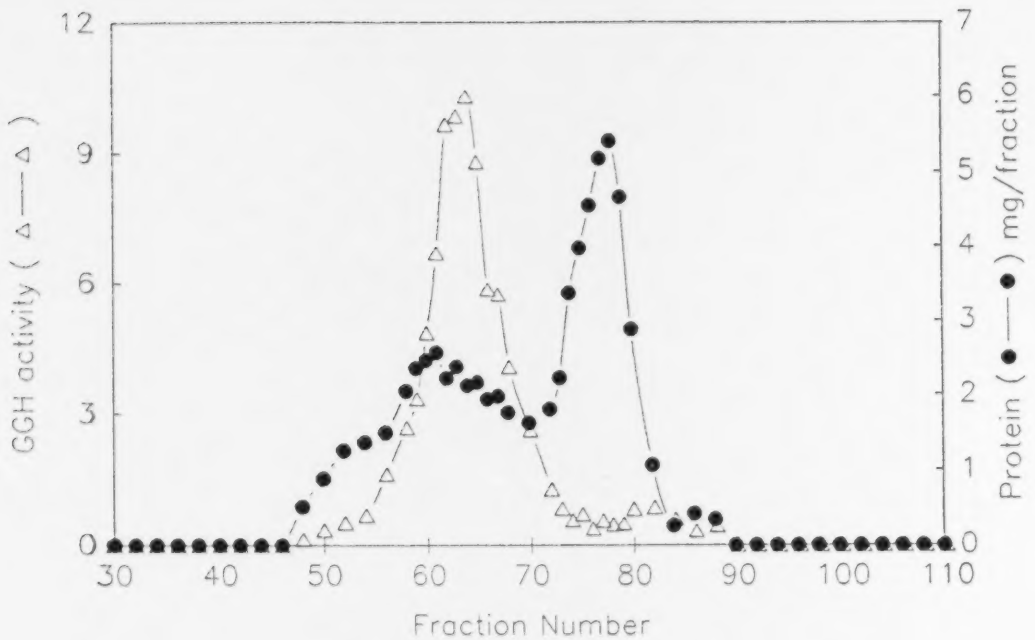


Fig. 9. Gel filtration of GGH activity.
After concentration of Step 4 protein by ultra-filtration, 12 ml were applied to a column of Sephacryl S 200 (see Materials and Methods). Fractions (5 ml) were collected and assayed as in Fig. 8.

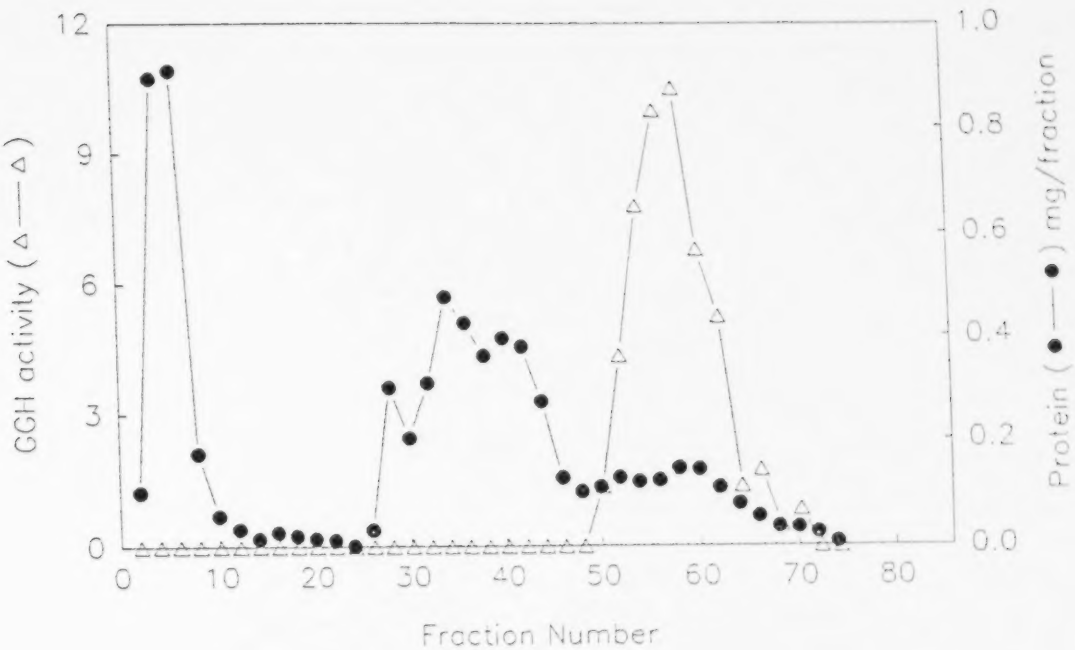


Fig. 10. Affinity chromatography on Matrex Green A. 25 ml of Step 5 protein from 1-day cotyledons were applied to a column of Matrex Green A and GGH protein was eluted using a linear NaCl gradient after collection of fraction 17 (see Materials and Methods). Fractions (6 ml) were collected and assayed as in Fig. 8.

kD, was accompanied by three minor bands of greater mobility (data not shown). Heparin agarose chromatography of this concentrated step 6 protein (Fig. 11) resulted in further protein fractionation. SDS-polyacrylamide electrophoresis of the GGH-active fractions after this step revealed two protein bands migrating with M_r values of *ca* 27 kD and 23 kD respectively plus a minor band with a M_r of about 17 kD (data not shown).

3-3 GGH Stability and Reaction Requirements

After Matrex Green A chromatography, GGH activity of 1 day cotyledons was relatively unstable when stored at 4°C for 9 days in 100 mM Na phosphate buffer (pH 6.0) containing 20% glycerol. The enzyme of 6 day cotyledons, purified to step 6 followed by heparin agarose chromatography, was more stable under these storage conditions, retaining about 85% of the initial activity after 9 days. These losses at 4°C are comparable to those reported for the purified hepatic hydrolase (Silink et al, 1975). Unlike the GGH proteins of animal tissues (Elsenhans et al, 1984, Chandler et al, 1986), the pea hydrolase was labile when preincubated at elevated temperatures. Thus preincubation of step 6 protein for less than 20 min at 60°C destroyed all GGH activity. On the other hand, pea GGH was stable when preincubated for 1 hr at 40°C and then assayed at 37°C. Moreover, a short term assay at 45°C gave the highest GGH activity (Fig. 12).

The requirements for folylpolyglutamate hydrolysis were examined in assays of Step 7 GGH protein. Hydrolysis of $PteGlu_3$ and $PteGlu_5$ were both maximal at pH 6.0 as noted earlier (Fig. 7). In this regard, there was no evidence for optimal hydrolysis of the longer-chained conjugate under alkaline conditions as previously noted for crude extracts of cabbage leaves (Tamura et al, 1972). Under the standard assay conditions, release of label from [^{14}C]-$PteGlu_3$ was a linear function of time for *ca* 45 min at 37°C (Fig. 13). The rate of product formation with this substrate was also a linear

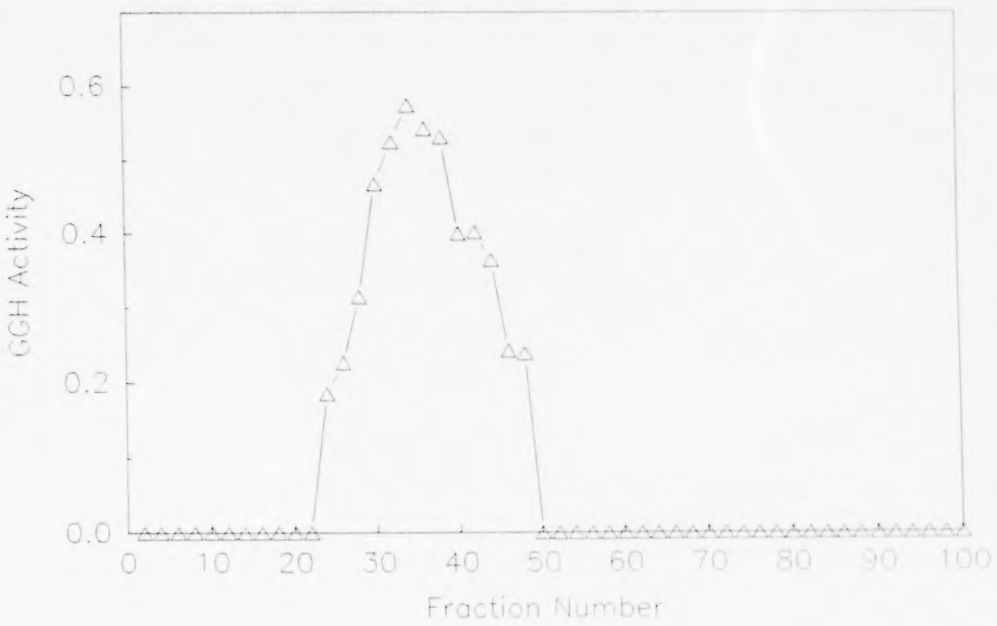


Fig. 11. Chromatography of GGH protein on heparin agarose.

Step 6 protein (5 ml) was diluted in 20 ml of Buffer A and applied to a 1.5 X 10 cm column of heparin agarose. A linear NaCl gradient (200 ml of Buffer A to 200 ml of Buffer A containing 0.6 M NaCl) was applied with the collection of fraction 1. Fractions (3 ml) were collected and assayed for GGH activity. The protein content of these fractions was below the detection limits (1 ug protein/ml) of the Bradford microassay. GGH activity is expressed as in Fig. 8.

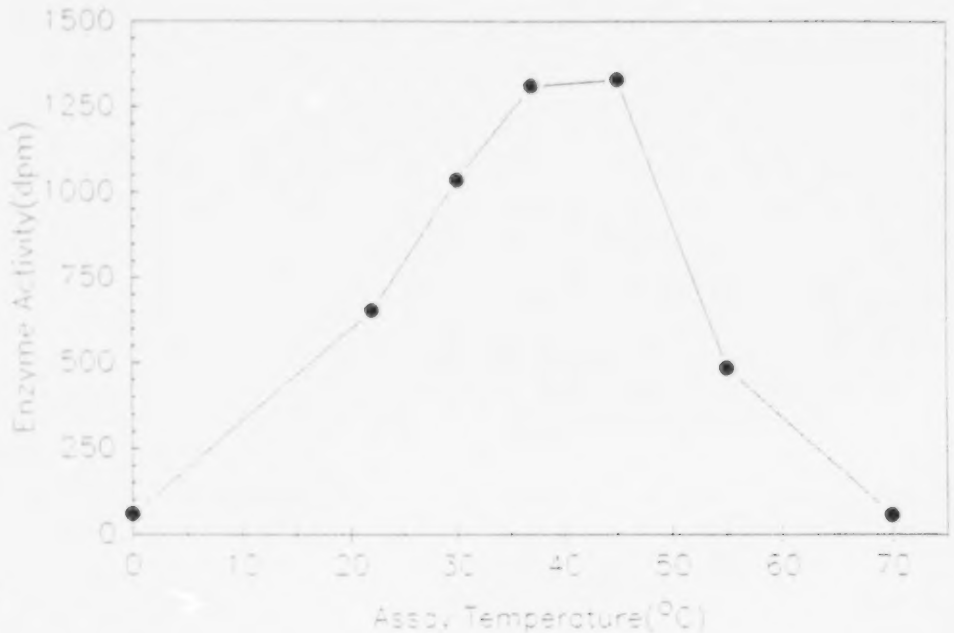


Fig. 12. Effect of assay temperature on GGH activity. The reaction systems with step 4 GGH protein were pre-incubated at the indicated temperatures for 2-3 min. The reaction was then initiated by the addition of 10 nmoles substrate $PteGlu_3$. After 15 min incubation at the indicated temperatures, the reaction was terminated as described in the Materials and Methods. 1 ml of the fluid was counted for each reaction. Activity is expressed as dpm/ml of reaction product. Data are averages of two separate determinations.

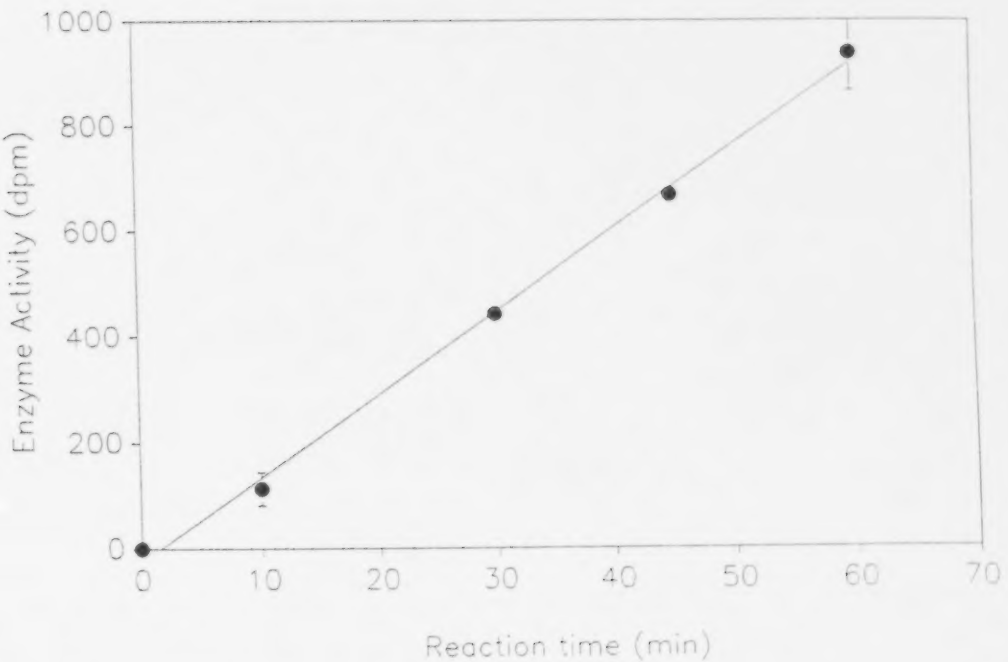


Fig. 13. Time course of GGH product formation.
Standard reaction systems containing 10 ug of Step 6 GGH protein were incubated at 37°C for various lengths of time. The reaction was terminated by the addition of 0.5 ml TCA as described in the Materials and Methods. 1 ml of the reaction fluid was counted after charcoal treatment (see Materials and Methods). Activity is expressed as dpm/ml of reaction product. Data are averages of two separate determinations.

function of protein concentration in assays containing 4 to 23 ug of step 6 protein (Fig. 14). Product formation was not affected by additions, to the reaction system, of BSA and glutamate (Table 5). However 2-mercaptoethanol (*ca.* 9 mM) resulted in *ca.* 50% inhibition of pea GGH activity. Incontrast, this compound stimulates mammalian plasma and pancreatic GGH activities (McGuire and Coward, 1984; Bhandari et al, 1990). The plant enzyme was also not inhibited by adding, to the 1 ml reaction system, 5 to 5000 ng of heparin (Table 5), a potent inhibitor of the hepatic enzyme (Silink et al, 1975).

3-4 Kinetic Studies of GGH

Kinetic studies were carried out with step 6 protein using $PteGlu_3$ and $H_4PteGlu_3$ as substrates. The apparent K_m values for these folates at pH 6.0, as determined by Lineweaver-Burk plots (Figs. 15 and 16), were 24 and 36 uM, respectively. The apparent V_{max} values for these substrates were 0.114 umoles of $PteGlu_3$ hydrolyzed/min/mg protein and 4.5 umoles of $H_4PteGlu_3$ hydrolyzed/min/mg protein.

3-5 Inhibitors of GGH Activity

In agreement with Iwai and Nakagawa's 1957 study of soybean leaf GGH, the enzyme from pea cotyledons was strongly inhibited by Zn ions (Fig. 17). This is in contrast to the activation of some mammalian GGH proteins by this metal (Silink et al, 1975; McGuire and Coward, 1984; Chandler et al, 1986; Bhandari et al, 1990). Sensitivity was also shown to p-HMB (Fig. 18) which inhibits the intracellular but not the membrane-bound GGH proteins of human and various animal jejunal mucosae (Reisenauer et al, 1977; Wang et al, 1985). Preincubation with the protease inhibitor PMSF at 0.1 mM, which has no effect on pancreatic GGH activity (Bhandari et al, 1990), caused more than 50% inhibition of the pea enzyme (Fig. 17). On the other hand, the carboxypeptidase inhibitor MMGA (Whitehead et al, 1990), supplied at 0.04

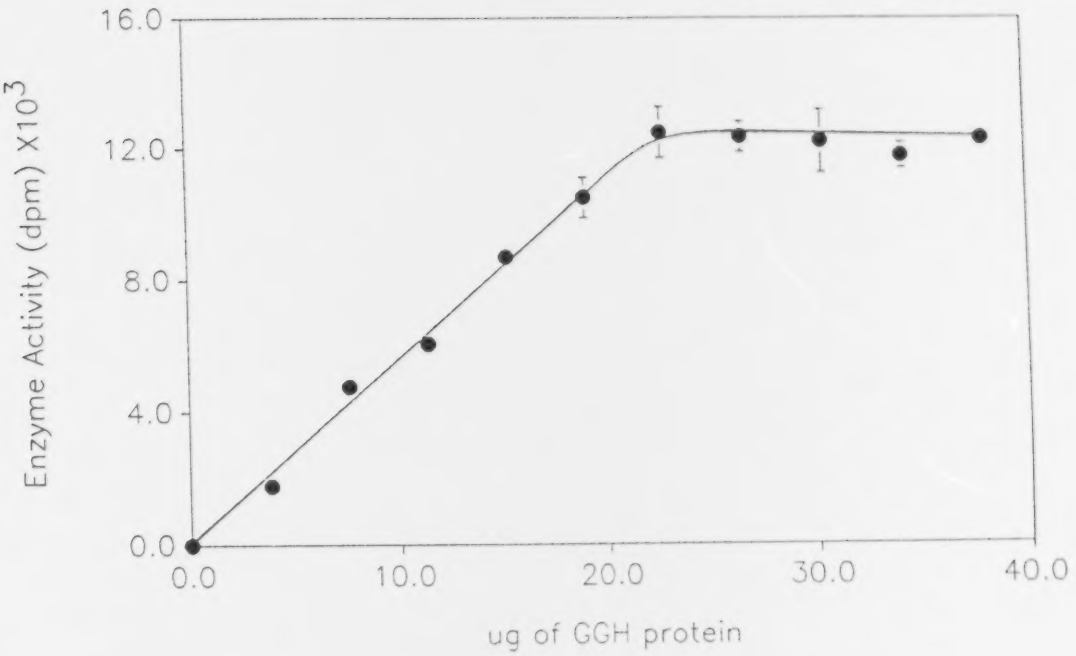


Fig. 14. Effect of GGH concentration on the hydrolysis of $PteGlu_3$.

0.8 ml standard assay systems containing 3.8-38 ug of step 6 protein were incubated at 37°C for 30 min and treated as described in the Materials and Methods. Results are the averages of two separate assays. Enzyme activity is expressed as dpm/reaction.

Table 5. The effects of heparin, glutamic acid, BSA and 2-ME on GGH activity.

Addition		1	2	3	4	5	6
Heparin	(ng)	0	5	25	40	50	5000
activity	(%)	100	98.2	100	100	100	100
Glu*	(uM)	0	30	50	70	90	120
activity	(%)	100	100	99.8	102	100.5	100
BSA**	(ug/ml)	0	60	70	90	100	120
activity	(%)	100	99.9	99.6	101	100.5	99.9
2-ME***	(mM)	0	1	3	7	9	15
activity	(%)	100	87.2	80.4	63.5	53.0	45.1

* Glu = L-glutamate. ** BSA = Bovine serum albumin. *** 2-ME = 2-mercaptoethanol. Data are averages of two separate determinations. GGH activity is expressed as a percentage of controls. GGH protein (step 6) was isolated from 6-day cotyledons using buffer A lacking 2-ME.

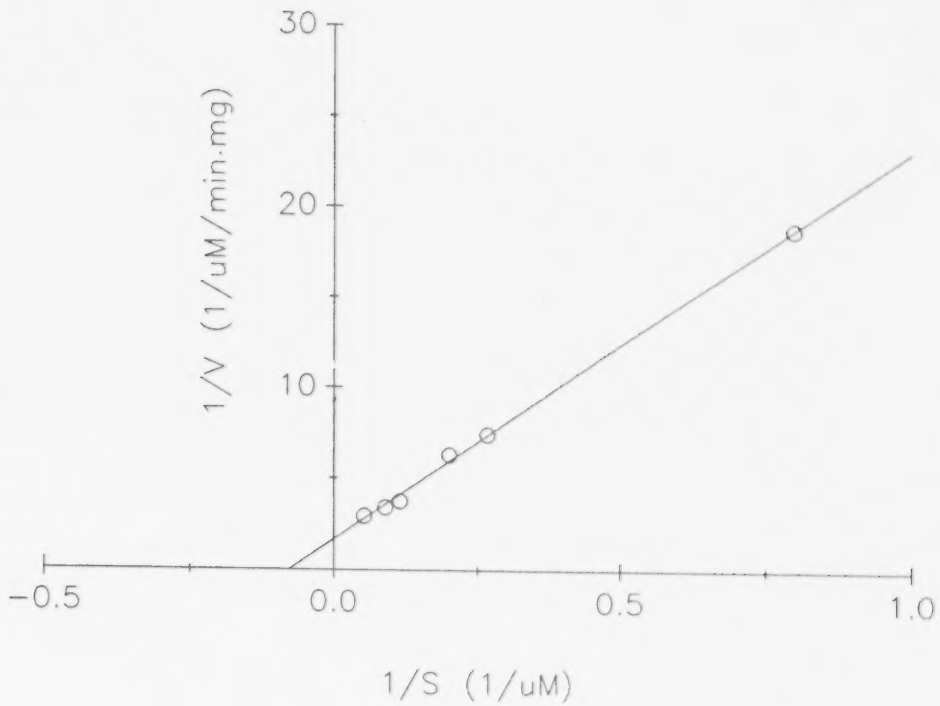


Fig. 15. Lineweaver-Burk plot for pea GGH using $PteGlu_3$ as substrate

Reaction systems containing 5.5 ug of Step 6 protein and various amounts of $PteGlu_3$ (1.25 to 18.75 uM) were incubated at 37°C for 30 min. The reactions were terminated and treated with charcoal as described in the Materials and Methods. Data are the averages of two separate assays. The apparent Km was calculated as 24 uM and Vmax was 114 nmole $PteGlu_3$ hydrolyzed/min/mg protein.

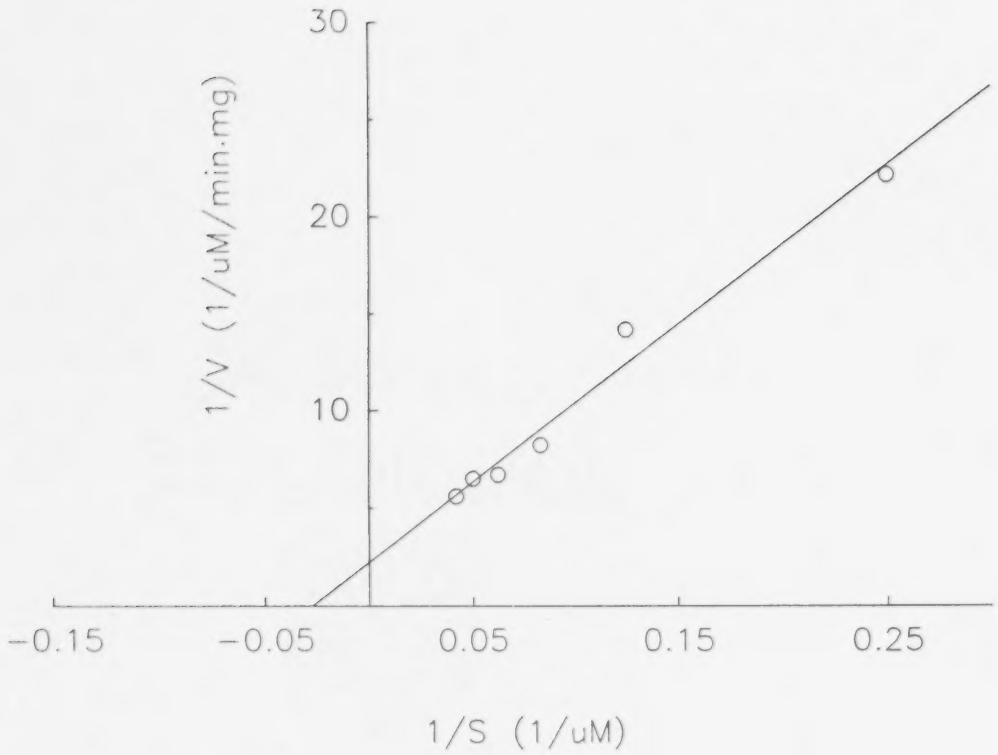


Fig. 16. Lineweaver-Burk plot for pea GGH using $H_4PteGlu_3$ as substrate

Reaction systems containing 2 ug of Step 6 protein and various amounts of $H_4PteGlu_3$ (4 to 24 uM) were incubated at 37°C for 30 min. The reactions were terminated and treated with charcoal as described in the Materials and Methods. Data are the averages of two separate assays. The apparent Km was calculated as 36 uM and the Vmax was 4.5 umole $H_4PteGlu_3$ hydrolyzed/min/mg protein.

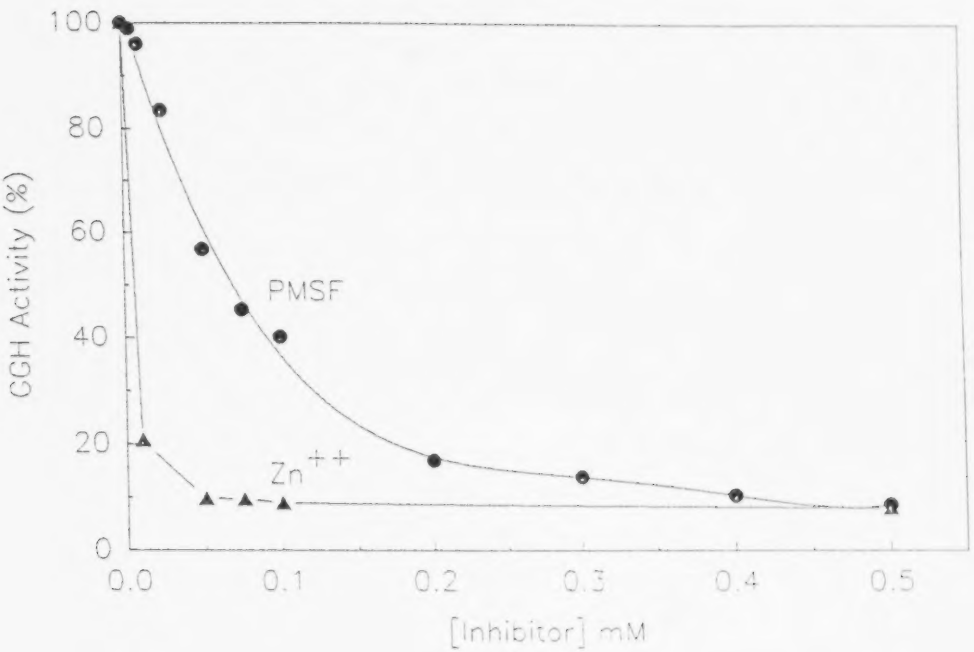


Fig. 17. Inhibition of GGH activity by PMSF and Zn ions. Samples of Step 6 protein were pre-incubated for 15 min at 37°C with varying amounts of PMSF (●) or zinc acetate (▲) as indicated. The GGH reaction was then initiated by addition of labelled $PteGlu_3$ (see Materials and Methods) and terminated after 30 min. Controls lacking PMSF or zinc acetate had average specific activities of 18.5 nmoles $PteGlu_3$ hydrolyzed/min/mg protein. Data are the averages of two separate determinations.

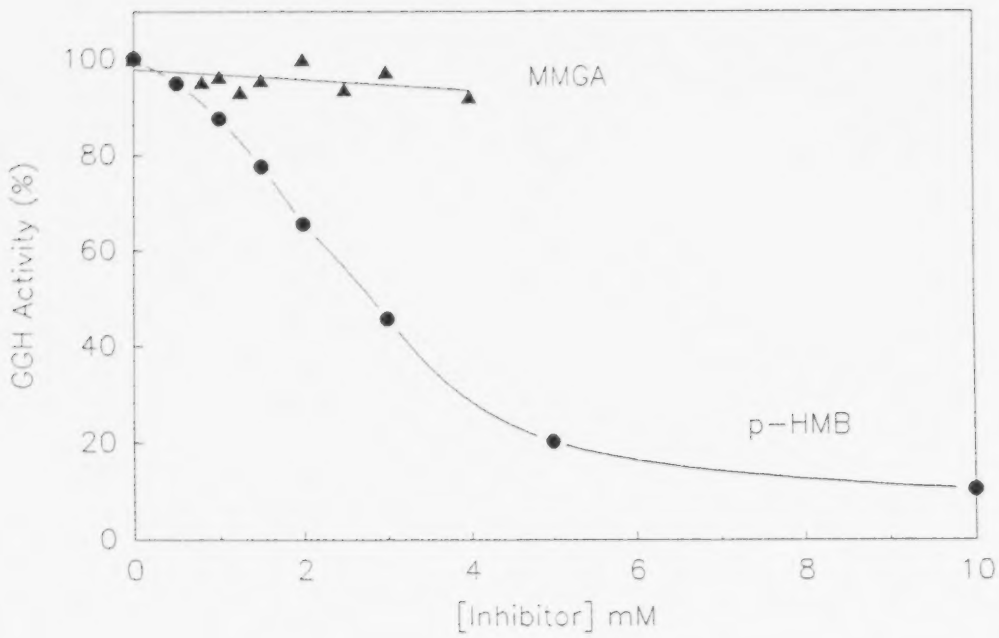


Fig. 18. The effect of MMGA and p-HMB on GGH activity. Samples of Step 6 protein were pre-incubated as in Fig. 17, with varying amounts of MMGA (▲) and p-HMB (●). Data are the averages of two separate determinations. Other details as in Fig. 17.

to 4 mM, failed to inhibit pea GGH activity (Fig. 18).

3-6 Molecular Weight of Pea Cotyledon GGH

Gel filtration of GGH activity on a calibrated column of Sephacryl S 200 gave an apparent M_r of *ca* 55 kD (Fig. 19). Comparable values for the GGH proteins of *E. coli*, chicken liver, chicken pancreas and rat pancreatic juice are 56 kD, 57.5 kD, 52 kD and 60 kD respectively (McGuire and Coward, 1984). GGH proteins of larger molecular size have been reported from other mammalian sources (McGuire and Coward, 1984; Silink et al, 1975; Elsenhans et al, 1984; Chandler et al, 1986).

3-7 Analysis of Reaction Products

The folate products derived from the hydrolysis of [^{14}C]-$PteGlu_3$ and $PteGlu_5$ were examined by HPLC as described in the Materials and Methods. This technique separates $pABAGlu_n$ derivatives according to their chain lengths (Fig. 20). Both $PteGlu_3$ and $PteGlu_5$ were converted to PteGlu (Fig. 21A, B). Small amounts of the diglutamate were formed from $PteGlu_5$ but not from $PteGlu_3$. These results indicated that GGH of pea cotyledon is an endopeptidase. Analysis of non-folate product derived from [^{14}C]-$PteGlu_3$ hydrolysis (first peak of Fig. 4, see Materials and Methods) revealed a labelled compound whose elution from Dowex (acetate) column was distinct from that of authentic glutamate (Fig. 22). HPLC analysis of this product (Zheng et al, 1992) showed that the label co-chromatographed with authentic gamma-glutamyl dipeptide (R_t = 4.7 min). Acid hydrolysis of this product, followed by ion exchange chromatography (Fig. 22) resulted in a labelled compound that co-chromatographed with authentic glutamate.

3-8 Substrate Specificity of GGH

The substrate specificity of pea GGH was examined as summarized in Fig. 21.

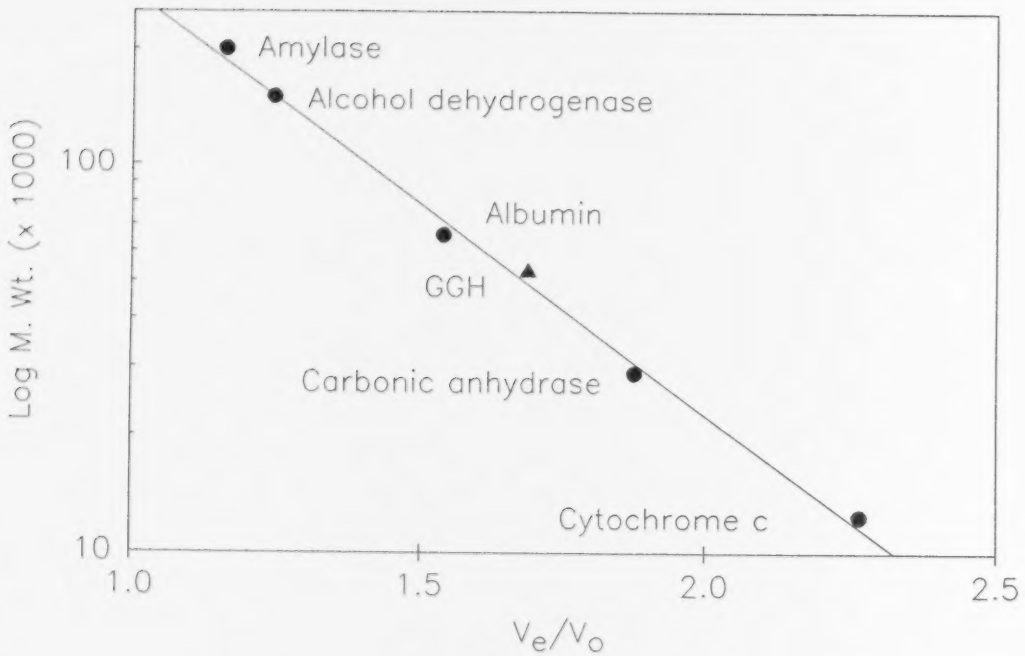


Fig. 19. Determination of molecular weight of GGH. Molecular weight standards were applied to a Sephacryl S 200 column (see Materials and Methods). The volumes required for elution of each protein (Ve) were determined by measuring absorbance at 280 nm. In the case of GGH, this volume was determined by the standard enzyme assay (see Materials and Methods). The apparent molecular weight of GGH, based on the average of five determinations, was calculated as 55 kD (range from 53.8 to 56 kD).

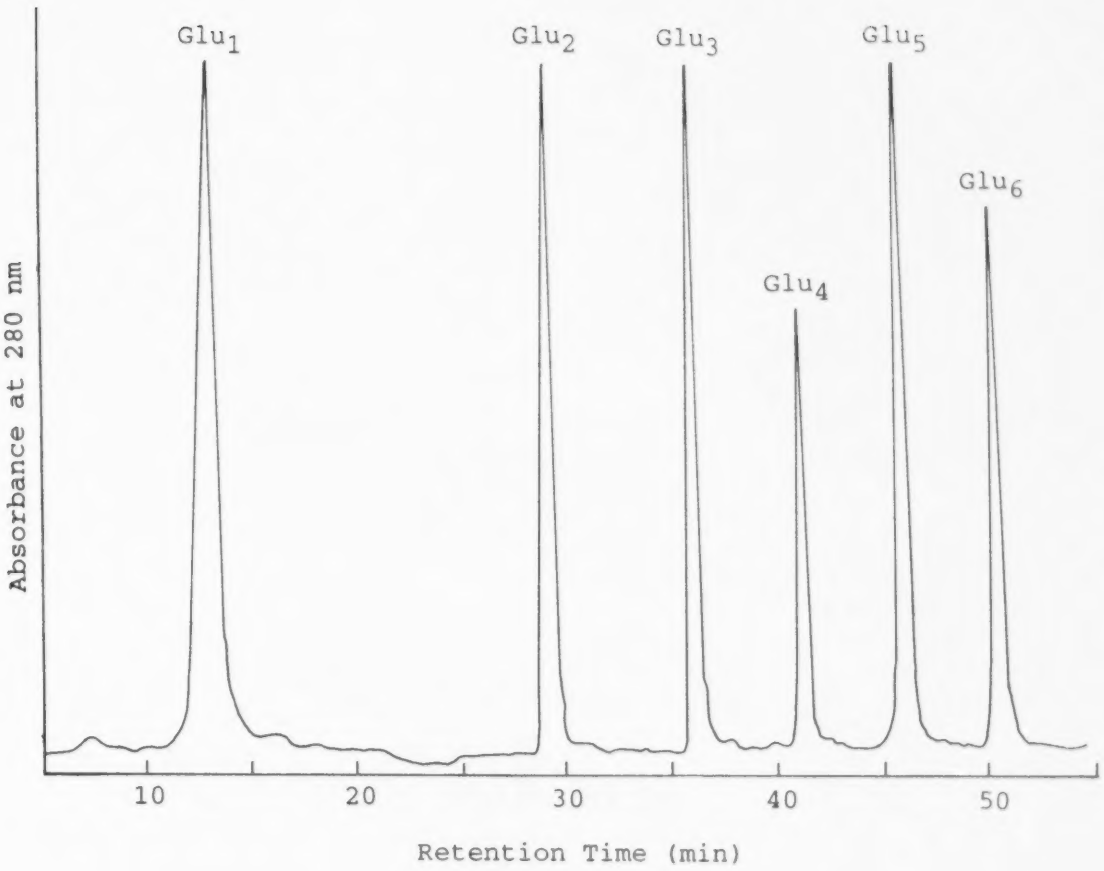


Fig. 20. Determination of folate chain lengths by HPLC. A 200 ul mixture of $pABAGlu_n$ standards (Dr. B. Schircks Laboratories, Jona, Switzerland) was injected and eluted from the column as described in the Materials and Methods. The retention times for $pABAGlu_n$ with 1 to 6 glutamyl residues were 12.4, 29.1, 35.9, 41.1, 45.9 and 50.4 min, respectively. Glutamyl chain lengths of folate products of GGH assay were also examined by this procedure following conversion to $pABAGlu_n$ derivatives (see Materials and Methods, also see Fig. 21).

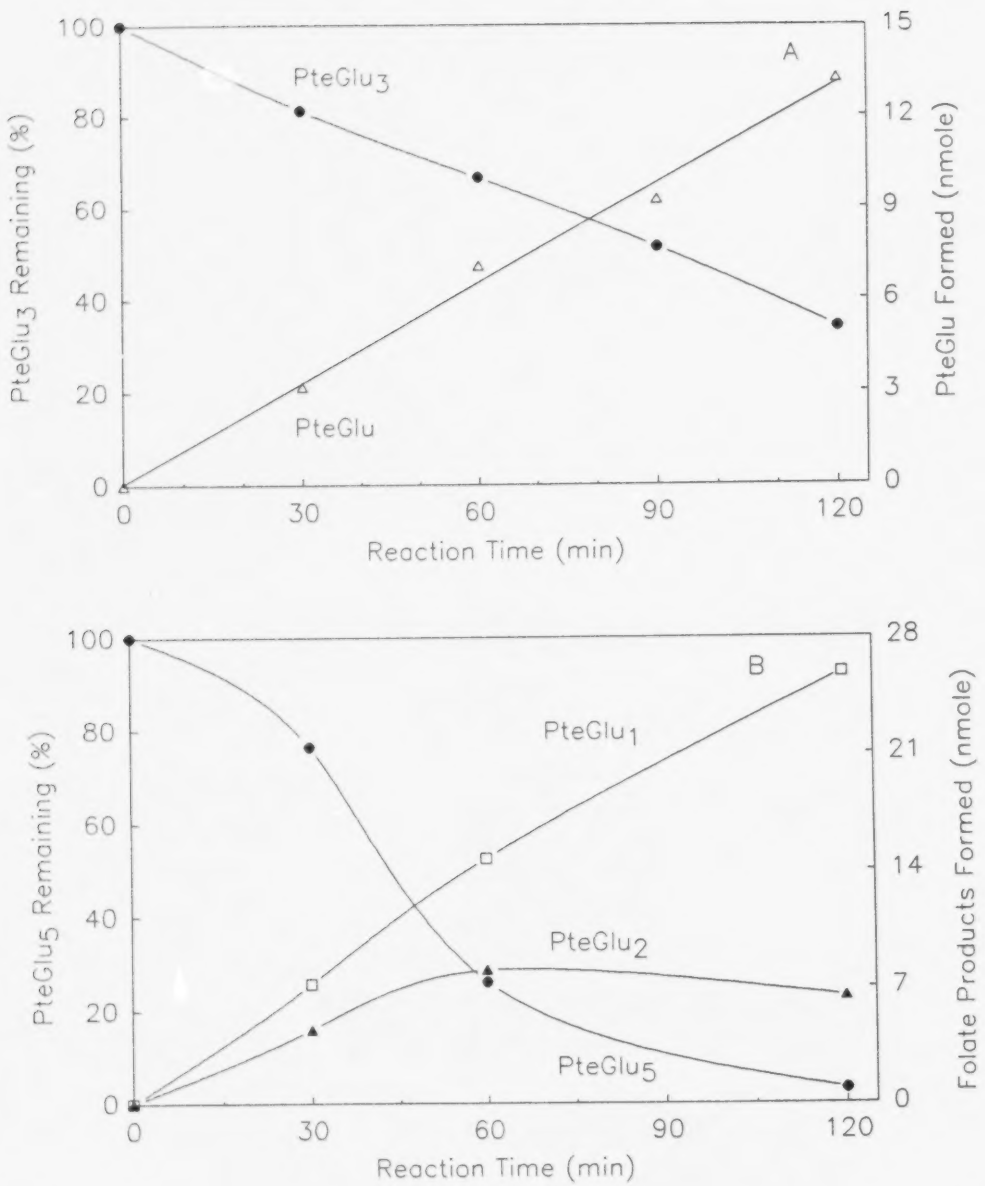


Fig. 21. Hydrolysis of $PteGlu_3$ and $PteGlu_5$.
Standard GGH reaction systems in duplicates contained:- A. 30 nmoles of $PteGlu_3$ and 1.2 ug of Step 6 protein; B. 30 nmoles of $PteGlu_5$ and *ca.* 0.65 mg of Step 3 protein. After incubation for various lengths of time, reactions were terminated with 2-mercaptoethanol and boiling followed by formation of azo dyes and HPLC analysis (see Materials and Methods).

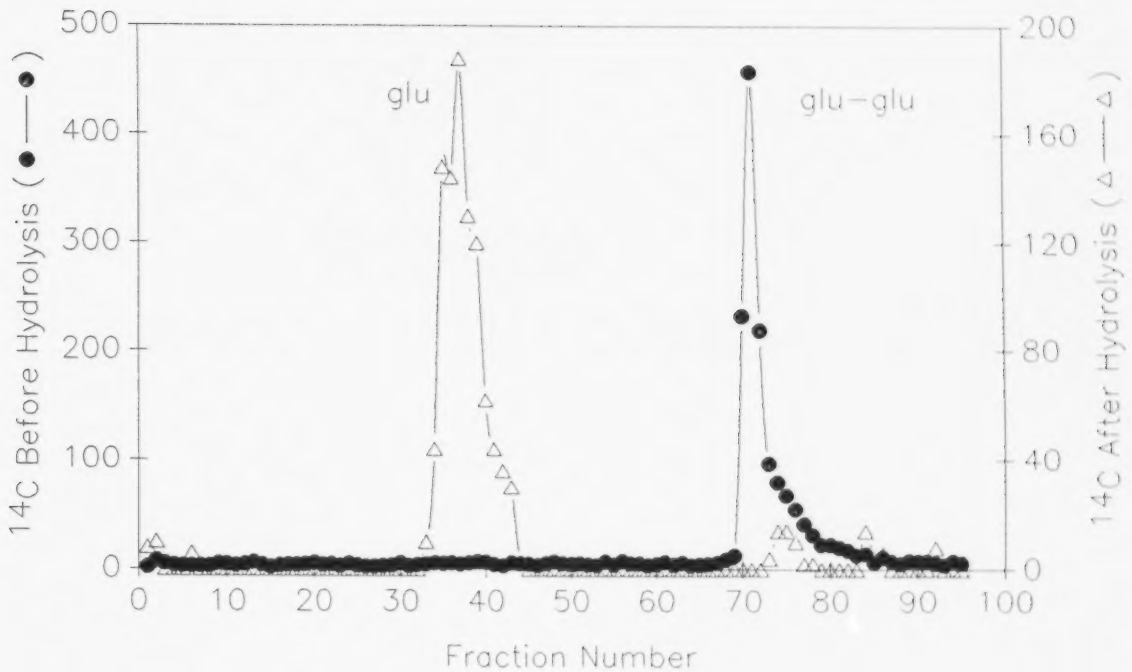


Fig. 22. Formation of gamma-glutamyl dipeptide after [^{14}C]PteGlu$_3$ hydrolysis.

Standard reaction systems (Step 4 protein) were incubated for 1 hr. Non- folate products were recovered and subjected to ion-exchange chromatography on Dowex 1-X8. Labelled fractions were pooled and incubated overnight at 120°C with 6 N HCl. After drying in vacuo, the product was re-chromatographed on Dowex (Δ). Radioactivities are expressed in dpm/0.1 ml before acid hydrolysis and in dpm/ml for acid hydrolyzed samples. In separate runs, authentic glutamate was collected in Fractions 30 to 45.

p-ABAGlu$_3$ was also hydrolyzed to the corresponding monoglutamate (Fig. 23) but the rate of this conversion was lower than for either of the folate substrates. The hydrolysis of [^{14}C]-PteGlu$_3$ was strongly inhibited by additions of PteGlu$_5$ to the reaction system (Fig. 24). In contrast, PteGlu and gamma-glutamyl tripeptide had little or no effect on the hydrolysis of [^{14}C]-PteGlu$_3$. The relatively small concentrations of PteGlu$_5$ needed to reduce [^{14}C]-PteGlu$_3$ hydrolysis implies that the pea enzyme may have greater affinity for the longer-chained substrate. A preferential cleavage of long-chained folylpolyglutamates has been described for the hydrolases of bovine liver (Silink et al, 1975) and human jejunal brush borders (Chandler et al, 1986).

In other assays, reactions systems that contained 100 nmoles of gamma-glutamyl tripeptide and were incubated for 1 hr at 37^{o}C did not liberate ninhydrin-positive products (Table 6). Furthermore, N-CBZ-phe-ala a substrate of yeast carboxypeptidase Y (Feronee et al, 1986) was not hydrolyzed by pea GGH (step 6 protein) in assays of 1 hr duration (Table 6).

3-9 Activity of GGH When Incubated with Protein-Bound Substrates

To examine the availability of protein-bound folate polyglutamates to purified GGH, experiments were carried out with DHFR. The latter enzyme was pre-incubated with [^{14}C]-PteGlu$_3$ or [^{14}C]-H$_4$PteGlu$_3$ for 15 min before the addition of GGH. The addition of DHFR (up to 0.075 units) to a standard reaction system containing 10 nmoles of substrate did not affect the hydrolysis of [^{14}C]-PteGlu$_3$ (Fig. 24). This is in agreement with the observation of Wang et al (1993), where the preincubation of DHFR with substrate did not affect the hydrolysis of PteGlu$_5$ by GGH obtained from H35 hepatoma cells. However, the addition of DHFR signifiacatly reduced the hydrolysis of a physiologically existing form of folate, [^{14}C]-H$_4$PteGlu$_3$ (Fig. 25).

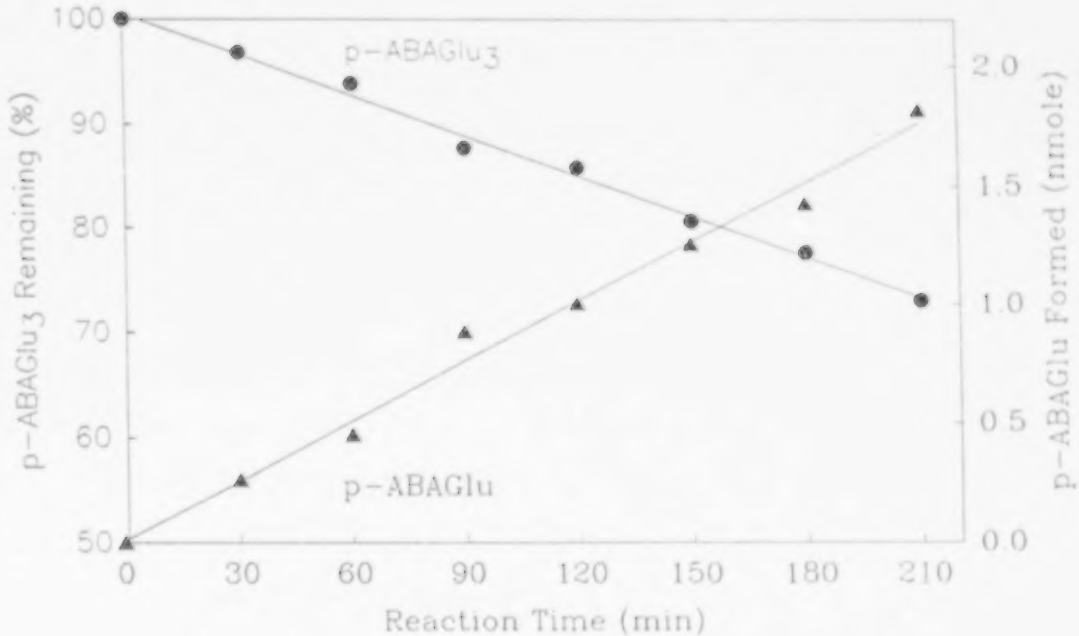


Fig. 23. Hydrolysis of pABAGlu$_3$

Standard GGH reaction system contained 10 nmoles of p-ABAGlu$_3$ and 1.2 ug of Step 6 protein. After incubation for various lengths of time, reactions were terminated with 2-mercaptoethanol and boiling followed by formation of azo dyes and HPLC analysis (see Materials and Methods). Data are the averages of duplicate assays.

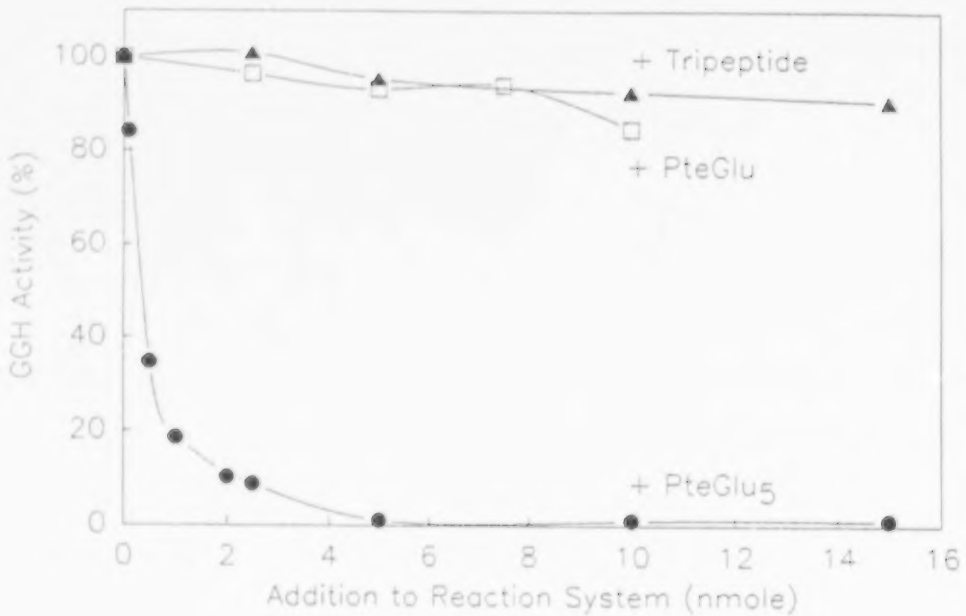


Fig. 24. The effect of gamma-glutamyl tripeptide, PteGlu and $PteGlu_5$ on the hydrolysis of $PteGlu_3$.

Standard reactions systems containing 10 nmoles of $PteGlu_3$ and varying amounts of gamma-glutamyl tripeptide (▲), PteGlu (□) or $PteGlu_5$ (●) were preincubated for 5 min at $37^{o}C$ followed by addition of 1.2 ug of Step 6 protein. The reactions were terminated, after 30 min incubation, as described in Materials and Methods. Data are the averages of three separate determinations.

Table 6. gamma-Glutamyl tripeptide and N-CBZ-phe-ala are not hydrolyzed by GGH protein.

Substrate	Net Change in Absorbance at 570 nm				
	Minutes of Incubation				
	0	10	20	45	60
1. Glutamyl tripeptide	n.d.	0.001	0.002	n.d.	0.003
2. N-CBZ-phe-ala	n.d.	0.040	0.030	0.030	0.030
3. N-CBZ-phe-ala + yeast carboxypeptidase*	n.d.	n.a	n.a	n.a.	1.560

*Yeast carboxypeptidase Y. n.d.= not detected; n.a.= not assayed.

1. Reactions systems (1 ml) contained 100 nmoles gamma-glutamyl tripeptide, 23.5 umoles of Na_3 citrate buffer (pH 5.5), 50 ul of Step 6 GGH.
2. Reaction systems (1 ml) contained 400 nmoles N-CBZ-phe-ala and buffer + enzyme as in 1.
3. Reaction systems (1 ml) contained substrate as in 2 + 25 umoles of citrate buffer and 5 ul of yeast carboypeptidase (Sigma).

After incubation at 30°C, ninhydrin reagent was added followed by boiling for 20 min. Absorbance was read at 570 nm.

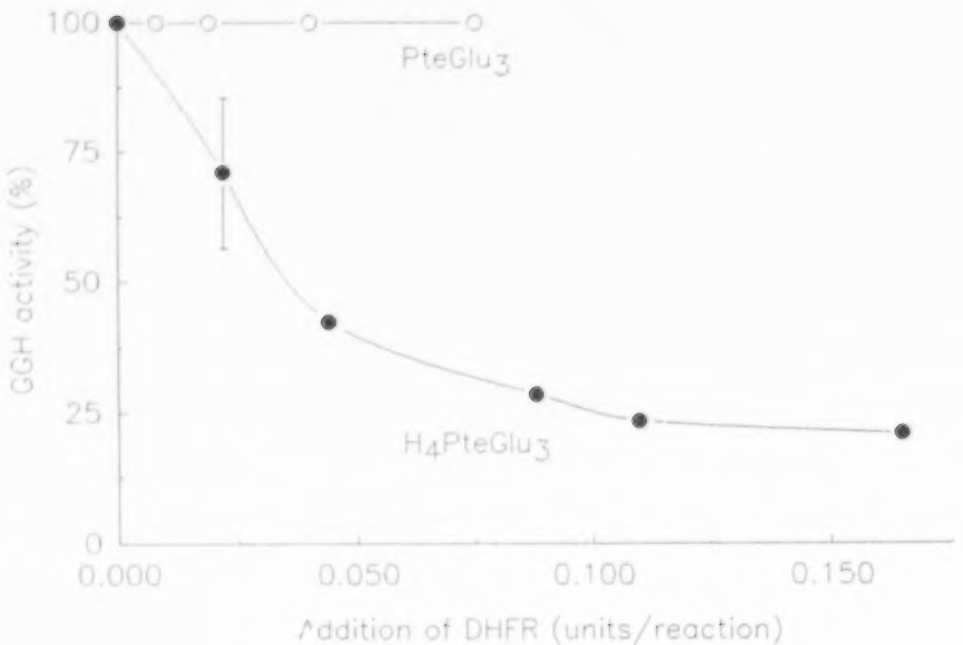


Fig. 25. Effect of DHFR on the hydrolysis of $PteGlu_3$ and $H_4PteGlu_3$.

In the standard reaction system, GGH was pre-incubated with various amounts of DHFR (Sigma, 1 unit DHFR converts 1 umole DHF to THF) for 15 min before initiating the reaction by the addition of the indicated folates. GGH activity is expressed as a percentage of controls lacking DHFR. Date are the averages of duplicate assays.

DISCUSSION

4-1 Purification of Pea Cotyledon GGH

The protocol used to purify GGH protein from pea cotyledons was initially modelled after that of Silink et al (1975), who had previously purified bovine hepatic gamma-glutamyl hydrolase to homogeneity. Pea cotyledon extracts were initially dialyzed after $(NH_4)_2SO_4$ precipitation of soluble protein. However, this step resulted in considerable losses of GGH activity. As a result, desalting was later achieved by passage of the extracts through Bio Gel P2 columns. When phenylagarose columns were examined for possible hydrophobic interactions with pea GGH protein, it was clear that binding did not occur. However, gel filtration chromatography on Sephacryl S 200 (Fig. 9) followed by non-specific affinity chromatography on Matrex Green A (Fig. 10) resulted in substantial purification of this protein (Table 4). When this step 6 protein was subjected to SDS-PAGE analysis, four silver-staining bands of protein were revealed. A further purification on heparin agarose, followed by SDS-PAGE analysis resulted in 3 silver-staining bands of approximately M_r 27, 23, and 17 kD, respectively. To determine if these polypeptides are all derived from native GGH, it may be necessary to carry out further affinity chromatography with a specific ligand, such as poly-gamma-glutamyl-Sepharose. This medium has been used very successfully in earlier work on rat intestinal mucosal GGH (Elsenhans et al, 1984).

4-2 Major Properties of Pea Cotyledon GGH

As noted in the Introduction, the importance of GGH activity in the hydrolysis of dietary folylpolyglutamates has resulted in the characterization of distinct membrane-associated and intracellular hydrolases from several mammalian tissues (McGuire and Coward, 1984; Reisenauer et al, 1977; Wang et al, 1985). The intracellular GGH proteins of mammals tend to show optimal activity at acidic pH (Reisenauer et al,

1977; Wang et al, 1985). These soluble enzymes also display endopeptidase action by producing PteGlu from $PteGlu_n$ (Elsenhans et al, 1984). Their activity is also strongly inhibited by p-HMB (Wang et al, 1985). In contrast, the membrane-associated GGH activities of mammalian cells are Zn-activated (Canton et al, 1989; Chandler et al, 1986; Wang et al, 1985) exopeptidases (Priest et al, 1982; Reisenauer et al, 1977) and are most active under neutral or alkaline conditions. This class of GGH proteins are not sensitive to inhibition by p-HMB (Reisenauer et al, 1977; Wang et al, 1985).

As in most other studies (Silink et al, 1975; Wang et al, 1985; Naughton et al, 1989; Chandler et al, 1991; Wang et al, 1993), the present work used $PteGlu_n$ as substrate in the standard assay of GGH activity. However, it is noteworthy that $PteGlu_n$ is not physiologically active in one-carbon metabolism until it is oxidized to $H_4PteGlu_n$. To examine the possible differences in the affinities shown for $PteGlu_n$ and $H_4PteGlu_n$, $H_4PteGlu_3$ was prepared using the method described by Stover and Schirch (1992). The apparent K_m values for $PteGlu_3$ and $H_4PteGlu_3$ were found to be 24 uM and 36 uM, respectively (Figs. 15, 16), values that are in the physiological range for endogenous folate concentrations (Shane, 1989). These results show that GGH from pea cotyledons is capable of hydrolyzing $H_4PteGlu_3$, a physiological folate substrate in plants (Besson et al, 1993; Kirk et al, 1993).

The present studies suggest that pea cotyledon GGH is a soluble protein (Table 3) with properties like those of the corresponding intracellular mammalian enzyme. In this regard, hydrolysis of $PteGlu_3$ was optimal at pH 6 but was strongly inhibited by Zn ions (Fig. 17) and p-HMB (Fig. 18). It is also concluded that this plant GGH is an endopeptidase as $PteGlu_3$ was converted to PteGlu (Fig. 21A) and gamma-glutamyl dipeptide (Fig. 22). Also consistent with this conclusion is the major formation of PteGlu from $PteGlu_5$ without accumulations of the pteroyl tri- or tetraglutamates (Fig. 21B).

Despite these basic similarities to the intracellular mammalian enzyme, the pea

hydrolase appeared to display a subunit construction when subjected to SDS-PAGE analysis. Further work with purer samples is needed to establish this point as there are reports, from research on animal tissues, of monomeric, soluble GGH proteins (McGuire and Coward, 1984; Elsenhans, 1984) and of oligomeric, membrane-associated GGH activities (McGuire and Coward, 1984; Chandler et al, 1986).

In common with the GGH of bovine liver (Silink et al, 1975), the enzyme from pea cotyledons appeared to display substrate specificity for substrates containing gamma-glutamyl peptide bonds. In this regard, the cotyledon GGH failed to hydrolyze N-CBZ-phe-ala, an alpha-amino peptide substrate of carboxypeptidase Y. The hepatic GGH and that from an H35 hepatoma cell line (Wang et al, 1993), are capable of hydrolyzing gamma-glutamyl peptide as substrate. This was not the case for pea GGH where an apparent requirement for the p-aminobenzoyl moiety of the substrate was indicated (Fig. 21, Table 6). Furthermore, the rate of hydrolysis catalyzed by pea GGH was enhanced when the substrate contained a pteridine moiety. Thus $PteGlu_3$ was more readily hydrolyzed than $p\text{-}ABAGlu_3$ (Fig. 21A, Fig. 23). In agreement with work on previously characterized GGH proteins (McGuire and Coward, 1984), that from pea cotyledons displayed a preference for relatively long-chained pteroylpolyglutamate substrates (Fig. 21). In this respect, $PteGlu_5$ strongly inhibited the hydrolysis of labelled $PteGlu_3$ (Fig. 24) suggesting that bound, highly conjugated folates were more amenable to hydrolytic attack.

4-3 The Possible Regulation of Pea Cotyledon GGH Activity

Pea cotyledons accumulate polyglutamates during early seedling growth (Roos and Cossins, 1971; Chan et al, 1986) and GGH activity increases from day 1 to day 5 (Table 2). During the early stages of seedling growth (Imeson and Cossins, 1991a,b), crude extracts of the cotyledons contain significant FPGS activities (ca. 2.5 nmol glutamate incorporated/hr/cotyledon) but these are significantly lower than the GGH

activities shown in Table 2. These enzymes do not appear to occupy different cellular compartments as most of the cotyledonary FPGS (Chan et al, 1986) and GGH (Table 3) activities are associated with the cytosolic fraction after differential centrifugation.

There is evidence from work on mammalian cells that folylpolyglutamates may not be accessible to hydrolase activity due to their binding to soluble folate binding proteins (Priest et al, 1982; Kane and Waxman, 1989; Henderson, 1990) or by their complexing with enzymes of folate metabolism. It should be noted that there appear to have been no reports of specialized folate-binding proteins in plants. However, by analogy with other biological systems (Schirch and Strong, 1989) it might be assumed that a significant part of the conjugated folate pool in pea cotyledons will be associated with folate-dependent enzymes. Such an association may protect these folates from hydrolysis by GGH. In this respect, the hydrolysis of $H_4PteGlu_3$ was significantly reduced when homogeneous DHFR protein was added to the standard GGH reaction system (Fig. 25). On the other hand, it is conceivable that GGH activity may be regulated during the early stages of germination so that this protein has little ability to attack endogenous folylpolyglutamates. In this regard, several legume species including pea, have been reported to contain GGH inhibitors (Krumdieck et al, 1973; Krumdieck et al, 1974; Bhandari and Gregory, 1990). These inhibitors are heat-activated organic, non-protein molecules. Their potential to selectively regulate GGH activity is not clear but appears to warrant more detailed investigation.

4-4 Some Possible Roles of GGH Activity in Folate and Antifolate Metabolism

An extensive hydrolysis of polyglutamates by GGH activity during germination is hard to visualize. Firstly, pea cotyledons accumulate these derivatives during early seedling growth (Roos and Cossins, 1971; Chan et al, 1986). Secondary, polyglutamates are the preferred substrates of many folate-dependent enzymes (Shane, 1989). Thus the active one-carbon metabolism of this tissue probably centres on a small

pool of C_1 substituted folylpolyglutamates. By analogy with other biological systems it follows that cotyledonary GGH activity may have the following roles. First, in response to various physiological demands of the developing seedling, this enzyme may alter folate metabolism by partially hydrolyzing polyglutamates to derivatives of shorter, specific chain length that are more suitable substrates for different folate-dependent enzymes (Krumdieck et al, 1977). Second, the enzyme may have roles related to the compartmentation of one-carbon metabolism (Appling, 1991). In this sense, transport of folates across organellar or cellular membranes is known to require the hydrolysis of folypolyglutamates to the corresponding monoglutamate derivatives (Halsted, 1989). This process is catalyzed by GGH and may therefore be regulated by the level of this hydrolase. Third, GGH activity may play an important role in the metabolism of conjugated antifolates such as methotrexate polyglutamates (Sur et al, 1986; Barrueco et al, 1992; Sikora et al, 1992; Wang et al, 1993). There has been recent interest in this role of mammalian GGH and it is evident that increased expression of these proteins can result in resistance to polyglutamable antifolates like methotrexate (Sur et al, 1986; Whitehead et al, 1990).

4-4-1 The Possible Regulation of Folylpolyglutamates Chain Length by Gamma-Glutamyl Hydrolase

As cellular folates are almost entirely folylpolygluta-mates and folate-dependent enzymes usually have greatest affinity for these derivatives (Strong and Schirch, 1989), it is clear that they should be considered the normal substrates of one-carbon metabolism. In addition, a failure to synthesize folylpolyglutamates leads to an inability to retain cellular folates and to synthesize products of one-carbon metabolism in sufficient quantities to support growth (Shane, 1989). The advantages of folyl- or antifolylpoly-glutamate have primarily been explored by assessing their substrate or inhibitory activity for purified enzymes of one-carbon metabolism.

Earlier studies of substrate specificity revealed the marked preference for polyglutamates by the folate-dependent enzymes of the purine cycle, of glycine-serine interconversion and C_1-THF synthase (Shane, 1989). However, comparable enzymes of the methionine and thymidylate cycles displayed a very different specificity for folylpolygluta-mates. For example, mammalian thymidylate synthases preferentially use 5,10-CH_2=H_4PteGlu$_6$ over the corresponding monoglutamate when both substrates are present at equivalent V_{max}/K_m ratios (Kisliuk et al, 1981). Methotrexate polyglutamates are potent inhibitors of human thymidylate synthase, with K_i values decreasing from 13 uM for the monoglutamate to 50 nM for the pentaglutamate (Allegra et al, 1985). On the other hand, the second enzyme of the thymidylate cycle, DHFR, does not show a marked preference for folylpolyglutamate substrates or inhibitors (Coward et al, 1974). These differences among the principal enzymes of one-carbon metabolism could provide the basis for an indirect regulation of these pathways by gamma-glutamyl hydrolase. Thus under different physiological and nutritional conditions, GGH might alter the glutamyl chain length of cellular folates and affect the flux of one-carbon units through the different reactions of one-carbon metabolism. Direct evidence for such an involvement of GGH has been provided by cultured human lymphocytes and fibroblasts (Whitehead et al, 1987). In these systems, MMGA, a specific inhibitor for the GGH with a neutral pH optimum, causes an accumulation of methotrexate polyglutamates. A similar role for plant GGH can not be firmly established until more is known about the folylpolyglutamate specificities of plant folate-dependent enzymes. Based on recent work in this area (Kirk et al, 1993; Besson et al, 1993) it seems reasonable to conclude that changes in the availability of long-chained folylpolyglutamates, due to hydrolysis by GGH, would affect the synthesis of glycine, serine and purines in pea seedlings.

4-4-2 Control of Cross Membrane Folate Transport by Gamma-Glutamyl

Hydrolase Activity

Despite the general belief that activated one-carbon units are independently generated in both the mitochondria and the cytoplasm, a recent study has demonstrated that folate metabolism is highly integrated between different cellular compartments (Barlowe and Appling, 1988). Therefore, transport of folate across membranes plays an important role in the regulation of folate distribution. Limited transpport could result in folate becoming a limiting factor for the synthesis of cellular constituents under certain physiological conditions. Jejunal uptake of folylpolyglutamate has been proposed as a two-step process (Halsted, 1991). Following the hydrolysis of $PteGlu_n$ by brush border GGH, the product, PteGlu is transported across membrane by an active, saturable, and pH-dependent process (Mason, 1990). This mechanism was also demonstrated in MA 104 cells (Kamen et al, 1988), a non-intestinal cell system, where the transported PteGlu was found to be converted to $PteGlu_n$ in the cytosol. Evidence for an intercompartmental pathway was found in rat liver where mitochondrial folate-mediated reactions generated one-carbon units for utilization in cytoplasmic processes (Barlowe and Appling, 1988). Because mammalian folates circulate exclusively in the monoglutamyl form, it is likely that the intracellular GGH is required to convert intracellular folylpolyglutamate to monoglutamate forms before further cross-membrane transport can take place. In this regard, pea cotyledon GGH cleaves $PteGlu_3$ and $PteGlu_5$ directly to PteGlu (Fig. 21A, B), which would appear to be the most efficient way to remove the gamma-glutamyl tail and generate pteroylmonoglutamate for possible transport. However, the current scarcity of information on this aspect of plant folate metabolism leaves little support for such a role of pea GGH as a regulator of cross-membrane folate transport. As higher plants synthesize folate *de novo* , there may be very limited transport of folates between individual cells. It is clear that more work on the compartmentalization of higher plant folate metabolism is needed to determine whether polyglutamates are hydrolyzed prior to movement between the cytosol and

various organelles that support vital, folate-dependent reactions.

4-4-3 GGH Activity and Methotrexate Resistance

In addition to the role of GGH in the nutritional uptake of folates, this enzyme now has an acknowledged role in methotrexate resistance (Sur et al, 1986; Whitehead et al, 1988; Barrueco et al, 1992). Methotrexate treatment stimulates an increase in GGH activity, an observation first made by Sur et al (1986) using Krebs ascites cells. The increase in GGH activity was clearly dose- and time- dependent. This result was supported by later studies of an Ehrlich Ascites Carcinoma cell line (Sikora et al, 1992). In other studies, H35 hepatoma cells, resistant to the antifolate 5,10-dideazatetrahydrofolate, have six-fold higher levels of GGH protein (Wang et al, 1993). Further, a methotrexate-resistant *Datura* cell line has GGH activities that are three times those of MTX-sensitive cells (Wu, personal communication). From all of these observations, it is clear that hydrolysis of polyglutamyl antifolates by GGH is a significant factor in conferring resistance to polyglutamable antifolates. These studies also emphasize the active involvement of GGH in modifying the polyglutamate status of folates and antifolates in living cells. There are, however, still significant gaps in knowledge of this subject.

4-5 Suggestions for Further Study

Many avenues for further research into this and related areas of plant folate metabolism may be suggested as a result of the present study. In the author's opinion, the following represent a sampling of the most challenging questions that merit future study in plant systems.

The compartmentation of individual folate-dependent enzymes is now emerging as an active research area (Shane, 1989; Appling, 1991). In this regard, evidence for an inter-compartmental pathway of one-carbon metabolism that involves the mitochondria

and cytosol of rat liver tissue has been proposed (Barlowe and Appling, 1988). On the other hand, the compartmentation of only a few folate-dependent enzymes in higher plant tissues has been investigated, and several laboratories have developed reliable methods for organelle isolation. Despite this, the studies of the correlation between organellar and cytosolic one-carbon metabolisms in plant tissues have not yet been attempted. Such studies could provide answers to some of the questions raised during the course of this thesis research. For examples, such research might provide insights into folate transport between plant organelles, the mechanism for such a transport, and the possible role of GGH in this process.

As mentioned in an earlier section, there appear to be a parallel increases in GGH activity and the net synthesis of folylpolyglutamates in pea cotyledons during early seedling growth. How the cotyledon tissue balances the activities of GGH and FPGS *in vivo* is unknown. Conceivably, GGH activity is regulated. In mouse cells, this is a proliferation-dependent enzyme (Sikora et al, 1992). Perhaps the level of expression of GGH protein varies at different stages of seedling development. All of these basic questions represent interesting topics for furture research.

As mentioned earlier, higher GGH activities have been reported for methotrexate-resistant cell lines (Sur et al, 1986; Wang et al, 1993). In addition, there is evidence, from work on H35R hepatoma cells, that an increase in GGH activity directly contributes to acquired resistance to antifolates including MTX (Rhee et al, 1993). However, related studies of these topics in higher plants have started only recently (Wu, personal communication). More work is needed to verify the relationships between increases in GGH activity and MTX resistance in these plant systems.

BIBLIOGRAPHY

AIMI, J., QIU, H., WILLIAMS, J., ZALKIN, H. and SIXON, J.E. 1990. *De novo* purine nucleotide biosynthesis: cloning of human and avian cDNAs encoding the trifunctional glycinamide ribonucleotide synthetase-aminoimidazole ribonucleotide synthetase-glycinamide ribonucleotide transformylase by functional complementation in *E. coli*. Nucleic Acids Research. 18:6665-6672.

ALLEGRA, C.J., DRAKE, J.C., JOLIVET, J. and CHABNER, B.A. 1985. Inhibition of phosphoribosylaminoimidazolecarboxamide transformylase by methotrexate and dihydrofolic acid polyglutamates. Proceedings of National Academy of Science, U.S.A. 82:4881-4885.

AMSDEN, G.W., KOWALSKY, S.F. and MORSE G.D. 1992. Trimetrexate for *Pneumocystis carinii* pneumonia in patients with AIDS. The Annals of Pharmacotherapy. 26.218-226.

APPLING, D.R. 1991. Compartmentation of folate-mediated one-carbon metabolism in eukaryotes. The FASEB Journal 5:2645-2651.

APPLING, D.R. and RABINOWITZ, J.C. 1985. Regulation of expression of the ADE3 gene for yeast C_1-tetrahydrofolate synthase, a trifunctional enzyme involved in one-carbon metabolism. The Journal of Biochemical Chemistry 260:1248-1256.

BANERJEE, R.V., HARDER, S.R., RAGSDALE, S.W. and MATTHEWS, R.G. 1990. Mechanism of reductive activation of cobalamin-dependent methionine synthase: an electron paramagnetic resonance spectroelectrochemical study. Biochemistry. 29:1129-1135.

BANERJEE, R.V. and MATTHEWS, R.G. 1990. Cobalamin-dependent methionine syntase. The FASEB Journal. 4:1450-1459.

BARLOWE, C.K. and APPLING, D.R. 1988. In vitro evidence for the involvement of mitochondrial folate metabolism in the supply of cytoplasmic one-carbon units. Biofactors. 1:171-176.

BARLOWE, C.K. and APPLING, D.R. 1990. Isolation and characterization of a novel eukaryotic monofunctional NAD-dependent 5,10-methylenetetrahydrofolate dehydrogenase. Biochemistry. 29:7089-7094.

BARREDO, J. and MORAN, R.G. 1992. Determinants of antifolate cytotoxicity: folylpolyglutamate synthetase activity during cellular proliferation and

development. Molecular Pharmacology 42:687-694.

BARRUECO, J.R., O'LEARY, D.F. and SIROTNAK, F.M. 1992. Metabolic turnover of methotrexate polyglutamates in lysosomes derived from S180 cells. The Journal of Biological Chemistry. 267:15356-15361.

BESSON, V., REBEILLE, F., NEUBURGER, M., DOUCE, R. and COSSINS, E.A. 1993. Effects of tetrahydrofolate polyglutamates on the kinetic parameters of serine hydroxymethyltransferase and glycine decarboxylase from pea leaf mitochondria. Biochemical Journal (in press).

BHANDARI, S.D. and GREGORY III, J.F. 1990. Inhibition by selected food components of human and porcine intestinal pteroylpolyglutamate hydrolase activity. American Jornal of Clinical Nutrition. 51:87-94.

BHANDARI, S.D., GREGORY III, J.F., RENUART, D.R. and MERRITT, A.M. 1990. Properties of pteroylpolyglutamate hydrolase in pancreatic juice of the pig. The Journal of Nutrition. 120:467-475.

BLAKLEY, R. L. 1984. Dihydrofolate reductase. in *Folates and Pterins*. (Blakley, R.L. and Benkovic, S.J., eds.) Vol 1. pp.191-253. Wiley, New York.

BOGNAR, A.L., OSBORNE, C., SHANE, B., SINGER,S.C. and FERONE, R. 1985. Folylpoly-gamma-glutamate synthetase-dihydrofolate synthetase: Coloning and high expression of the *Escherichia coli fol*C gene and purification and properties of the gene product. The Journal of Biological Chemistry. 260:5625-5630.

BOLLER, T. and KENDE, H. 1979. Hydrolytic enzymes in the central vacuole of plant cells. Plant Physiology. 63:1123-1132.

BOURGUIGNON, J., MACHEREL, D., NEUBURGER, M. and DOUCE, R. 1992. Isolation, characterization and sequence analysis of a cDNA clone encoding L-protein the dihydrolipoamide dehydrogenase component of the glycine cleavage system from pea leaf mitochondria. European Journal of Biochemistry 204:865-873.

CANTON, M.C., COTTER., B.M., CREMIN, F.M. and MORRISSEY, P.A. 1989. The effect of dietary zinc deficiency on pancreatic gamma-glutamyl hydrolase (EC 3.4.22.12) activity and on the absorption of pteroylpolyglutamate in rats. The British Journal of Nutrition. 62:185-193.

CHAN, P.Y., COFFIN, J.W. and COSSINS, E.A. 1986. *In vitro* synthesis of

pteroylpoly-gamma-glutamates by cotyledon extracts of *Pisum sativum* L. Plant Cell Physiology. 27:431-441.

CHANDLER, C.J., HARRISON, D.A., BUFFINGTON, C.A., SANTIAGO, N.A. and HALSTED, C.H. 1991. Functional specificity of jejunal brush-border pteroylpolyglutamate hydrolase in pig. American Journal of Physiology 260:G865-872.

CHANDLER, C.J., WANG, T.T.Y. and HALSTED, C.H. 1986. Pteroylpoly-glutamate hydrolase from human jejunal brush borders: Purification and characterization. The Journal of Biological Chemistry. 261:928-933.

CHEN, P., SCHULZE-GAHMEN, U., STURA, E.A., INGLESE, J., JOHNSON, D.L., MAROLEWSKI, A., BENKOVIC, S.J. and WILSON, I.A. 1992. Crystal structure of glycinamide ribonucleotide transformylase from *Escherichia coli* at 3.0 A resolution--A target enzyme for chemotherapy. Journal of Molecular Biology. 227:283-292.

CODY, V., LUFT, J.R., PANGBORN, W., TOY, J. and BOGNAR, A.L. 1992. Purification and crystallization of *Lactobacillus casei* folylpolyglutamate synthetase expressed in *Escherichia coli*. Journal of Molecular Biology. 224: 1179-1180.

COSSINS, E.A. 1980. One-carbon metabolism. in *The Biochemistry of Plants*. (Davies, D.D., ed.) Vol 2. pp. 365-418. Academic Press, New York.

COSSINS, E. A. 1984. Folates in biological materials. in *Folates and Pterins*. (Blakley, R.L. and Benkovic, S.J., eds.) Vol 1. pp.1-59. Wiley, New York.

COSSINS, E.A. 1987. Folate biochemistry and the metabolism of one-carbon units. in *The Biochemistry of Plants*. (Davies,D.D., ed.) Vol 11, pp. 316-353. Academic Press, New York.

COWARD, J.K., PARAMESWARAN, K.N., CASHMORE, A.R. and BERTINO, J.R. 1974. 7,8-Dihydropteroyl oligo-gamma-L-glutamates: synthesis and kinetic studies with purified dihydrofolate reductase from mammalian sources. Biochemistry. 13:3899-3903.

D'ARI, L. and RABINOWITZ, J.C. 1991. Purification, characterization, cloning, and amino acid swquence of the bifunctional enzyme 5,10-methylenetetrahydrofolate dehydrogenase/5,10-methenyltetrahydrofolate cyclohydrolase from *Escherichia coli*. The Journal of Biological Chemistry. 266:23953-23958.

DARWEN, C.W.E. and JOHN, P. 1989. Localization of the enzymes of fructan metabolism in vacuoles isolated by a mechanical method from tubers of Jerusalem Artichoke (*Helianthus tuberosus* L.). Plant Physiology. 89:658-663.

DAUBNER, S.C., SCHRIMSHER, J.L., SCHENDEL, F.J., YOUNG, M., HENIKOFF, S., PATTERSON, D., STUBBE, J. and BENKOVIC, S.J. 1985. A multifunctional protein possessing glycinamide ribonucleotide synthetase, glycinamide ribonucleotide transformylase, and aminoimidazole ribonucleotide synthetase activities in *de novo* purine biosynthesis. Biochemistry 24:7059-7062.

DEV, I.K. and HARVEY, R.J. 1978. A complex of N^5, N^{10}-methylenetetrahydrofolate dehydrogenase and N^5, N^{10}-methenyltetrahydrofolate cyclohydrolase in *Escherichia coli*, purification, subunit structure, and allosteric inhibition by N^{10}-formyltetrahydrofolate. The Journal of Biological Chemistry. 253:4245-4253.

EDMAN, U., EDMAN, J.C., LUDNGREN, B. and SANTI, D.V. 1989. Isolation and expression of the *Pneumocystis carinii* thymidylate synthase gene. Proceedings of National Academy of Science U.S.A. 86:6503-6507.

ELSENHANS, B., AHMAD, O. and ROSENBERG, I.H. 1984. Isolation and characterization of pteroylpolyglutamate hydrolase from rat intestinal mucosa. The Journal of Biological Chemistry. 259:6364-6368.

FEENEY, J. 1990. NMR studies of interactions of ligands with dihydrofolate reductase. Biochemical Pharmacology. 40: 141-152.

FERONE, R., SINGER, S.C. and HUNT, D.F. 1986. *In vitro* synthesis of alpha-carboxyl-linked folylpolyglutamates by an enzyme preparation from *Escherichia coli*. The Journal of Biological Chemistry. 261:16363-16371.

FLEMING, G.F. and SCHILSKY, R.L. 1992. Antifolates: the next generation. Seminars in Oncology. 19:707-719.

FREISHEIM, J.H. and MATTHEWS, D.A. 1984. The comparative biochemistry of dihydrofolate reductase. in *Folate Antagonists and Therapeutic Agents*. (Sirotnak, F.M. Burchall, J.J. and Ensminger, W.B. eds.). pp. 70-131. Academic Press. Orlando.

GALIVAN, J. 1979. Transport and metabolism of methotrexate in normal and resistant cultured rat hepatoma cells. Cancer Research. 39:735-743.

GALIVAN, J., JOHNSON, T., RHEE, M., McGUIRE, J.J., PRIEST, D. and KESEVAN, V. 1987. The role of folylpolyglutamate synthetase and gamma-glutamyl hydrolase in altering cellular folyl- antifolylpolyglutamates. Advances in Enzyme Regulation. 26:147-155.

GARROW, T.A., ADMON, A. and SHANE, B. 1992. Expression cloning of a human cDNA encoding folylpoly(gamma-glutamate) synthetase and determination of its primary structure. Proceedings of National Academy of Science U.S.A. 89:9151-9155.

GEROTHANASSIS, I.P., BIRDSALL, B., BAUER, C.J., FRENKIEL, T.A. and FEENEY, J. 1992. Nuclear magnetic resonance detection of bound water molecules in the active site of *Lactobacillus casei* dihydrofolate reductase in aqueous solution. Journal of Molecular Biology. 226:549-554.

GREGORY III, J.F. and CERDA, J.J. 1987. Comparison of pteroylpolyglutamate hydrolase (folate conjugase) from porcine and human intestinal brush border membrane. Comparative Biochemistry and Physiology. 88B:1135-1141.

GREGORY III, J.F., BHANDARI, S.D., BAILEY, L.B., TOTH, J.P., BAUMGARTNER, T.G. and CERDA, J.J. 1991. Relative bioavailability of deuterium-labeled monoglutamyl and hexaglutamyl folates in human subjects. American Journal of Clinical Nutrition 53:736-740.

HALSTED, C.H. 1975. The small intestine in vitamin B_{12} and folate deficiency. Nutrition Review. 33:33-37.

HALSTED, C.H. 1989. The intestinal absorption of dietary folates in health and disease. Journal of the American College of Nutrition. 8:650-658.

HALSTED, C.H. 1991. Jejunal brush-border folate hydrolase: A novel enzyme. Western Journal of Medicine. 155:605-609.

HAMLIN, J.L. and MA, C. 1990. The mammalian dihydrofolate reductase *locus*. Biochimica et Biophysica Acta. 1087: 107-125.

HENDERSON, G.B. 1990. Folate-binding proteins. Annual Review of Nutrition. 10:319-335.

HENIKOFF, S., KEENE, M.A., SLOANE, J.S. BLESKAN, J. HARDS, R. and PATTERSON, D. 1986. Multiple purine pathway enzyme activities are encoded at a single genetic locus in *Drosophila*. Proceedings of the National Academy of Science of U.S.A. 83:720-724.

HITCHINGS, G.H. 1989. Nobel Lecture in Physiology or Medicine: Selective inhibitors of dihydrofolate reductase. In Vitro Cell Developmental Biology. 25:303-310.

HOFFBRAND, A.V. and PETERS, T.J. 1969. The subcellular localization of pteroylglutamate hydrolase and folate in guinea pig intestinal mucosa. Biochimica et Biophysica Acta. 192:479-485.

HUENNEKENS, F.M., DUFFY, T.H. and VITOLS, K.S. 1987. Folic acid metabolism and its disruption by pharmacologic agents. National Cancer Institute Monographs 5:1-8.

HUENNEKENS, F.M., MATTHEWS, C.K. and SCRIMGEOUR, K.G. 1963. Preparation and properties of tetrahydrofolic acid. in *Methods in Enzymology*. (Colowick, S.P. and Kaplan, N.O., eds.) Vol 6 pp. 802-806. Academic Press. New York.

IMESON, H.C. and COSSINS, E.A. 1991a. Higher plant folypolyglutamate synthetase. I.--Purification, stability and reaction requirements of the enzyme from pea seedlings. Journal of Plant Physiology. 138:476-482.

IMESON, H.C. and COSSINS, E.A. 1991b. Higher plant folypolyglutamate synthetase. II.--Some major catalytic properties of the enzyme from *Pisum sativum* L. Journal of Plant Physiology. 138:483-488.

IMESON, H.C., ZHENG, L., and COSSINS, E.A. 1990. Folypolyglutamate derivatives of Pisum sativum L.: Determination of polyglutamate chain lengths by high performance liquid chromatography following conversion to p-amino-benzoylpolyglutamates. Plant Cell Physiology 310:223-231.

IVANETICH, K.M. and SANTI, D.V. 1989. Recent studies on thymidylate synthase. Annals New York Acacemy of Science. 569:201-210.

IVANETICH, K.M. and SANTI, D.V. 1990. Thymidylate synthase-dihydrofolate reductase in Protozoa. Experimental Parasitology. 70:367-371.

IWAI, K. 1957. Folic acid group in plant tissues VII. Some properties of folic acid conjugase in green leaves. Memoirs, Research Institute for Food Science. 13:1-9.

IWAI, K. and Nakagawa, S. 1957. Folic acid group in plant tissues VIII. Some properties of folic acid conjugase in green leaves. Memoirs, Research Institute

for Food Science. 13:10-16.

IWAI, K., FUJISAWA, Y. and SUZUKI, N. 1972. The accumulation of 5'-phosphoribosyl-5-amino-4-imidazolecarboxamide in folate-deficient pea seedlings and the enzymatic reaction in which the compound is involved. Agricultural and Biological Chemistry. 36:398-408.

JOHNSON, T.B., NAIR, M.G. and GALIVAN, J. 1988. Role of folypolyglutamate synthetase in the regulation of methotrexate polyglutamate formation in H35 hepatoma cells. Cancer Research. 48:2426-2431.

JOHNSON-FLANAGAN, A.M. and SPENCER, M.S. 1981. The effect of rotenone on respiration in pea cotyledon mitochondria. Plant Physiology. 68:1211-1217.

KALMAN, T.I., NAYAK, V.K., and REDDY, A.R.V. 1986. Selective inhibition of bacterial carboxypepticase G and pancreatic conjugase by 2-mercaptomethylglutaric acid. in *Chemistry and Biology of Pteridines 1989.* (Cooper, B.A., and Whitehead, V.M. eds.) pp.583-586. Walter de Gruyter, Berlin.

KALMAN, T.I. 1990. Polyglutamylation of folates and anti-folates as a target of chemotherapy. in Molecular Aspects of Chemotherapy. (Borowski, E. and Shugar, D. eds.) pp. 139-149. Plenum Press, New York.

KAMEN, B.A., WANG, M.T., STRECKFUSS, A.J., PERYEA, X. and ANDERSON, R.G.W. 1988. Delivery of folates to the cytoplasm of MA 104 cells in mediated by surface membrane receptor that recycles. The Journal of Biological Chemistry. 263:13602-13609.

KANE, M.A. and WAXMAN, S. 1989. Biology of disease--role of folate binding proteins in folate metabolism. Laboratory Investigation. 60:737-746.

KIRK, C.D., IMESON, H.C., ZHENG, L.L. and COSSINS E.A. 1993. The affinity of pea cotyledon 10-formyltetrahydrofolate synthetase for polyglutamate substrates. Phytochemistry (in press).

KISLIUK, R.L., GAUMONT, Y., LAFER, E., BAUGH, C.M. and MONTGOMERY, J.A. 1981. Polyglutamyl derivatives of tetrahydrofolate as substrates for *Lactobacillus casei* thymidylate synthase. Biochemistry 20:929-934.

KRUMDIECK, C.L. and BAUGH, C.M. 1970. A radioactive assay of pteroylpolyglutamate hydrolases. Analytical Biochemistry 35:123-131.

KRUMDIECK, C.L., CORNWELL, P.E., THOMPSON, R.W. and WHITE, W.E. Jr. 1977. Studies on the biological role of folic acid polyglutmates. in *Folic Acid.* (Food and Nutrition Board, ed.) pp. 25-42. National Academic Press. Washington. D.C.

KRUMDIECK, C.L., NEWMAN, A.J. and BUTTERWORTH, C.E. Jr. 1973. A naturally occurring inhibitor of folic acid cojugase (pteroyl-polyglutamyl hydrolase) in beans and other pulses. American Journal of Clinical Nutrition. 26:460-461.

KRUMDIECK, C.L., NEWMAN, A.J. and BUTTERWORTH, C.E. Jr. 1974. A naturally occurring inhibitor of folic acid cojugase (pteroyl-poly-gamma-glutamyl hydrolase) in beans and other pulses. Taxas Report in Biological Medicine. 32:820-821.

LEE, C.P., SEONG, B.L. and RajBHANDARY, U.L. 1991. Structural and sequence elements important for recognition of *Escherichia coli* formylmethionine tRNA by methionyl-tRNA transfoumylase are clustered in the acceptor stem. The Journal of Biological Chemistry. 266:18012-18017.

LICATO, N.J., COWARD, J.K., NIMEC, Z., GALIVAN, J., BOLANOWSKA, W.E. and McGUIRE, J.J. 1990. Synthesis of N-[N-(4-deoxy-4-amino-10-methylpteroyl)-4-fluoro-glutamyl]-gamma-glutamate, an unusual substrate for folylpoly-gamma-glutamate synthetase and gamma-glutamyl hydrolase. Journal of Medicinal Chemistry. 33:1022-1027.

LIN, S., ROGIERS, S. and COSSINS, E.A. 1993. Purification and properties of a pea cotyledon gamma-glutamyl hydrolase. Phytochemistry 32:1109-1117.

LU, Y.-Z., AIELLO, P.O. and METTHEWS, R.G. 1984. Studies on the polyglutamate specificity of thymidylate synthase from fetal pig liver. Biochemistry. 23:6870-6876.

MACHEREL, D. BOURGUIGNON, J. and DOUCE, R. 1992. Cloning of the gene (*gdcH*) encoding H-protein, a component of the glycine decarboxylase complex of pea (*Pisum sativum* L.) Biochemical Journal. 286:627-630.

MacKENZIE, R.E. 1984. Biogenesis and interconversion of substituted tetrahydro-folates. in *Folates and Pterins.* (Blakley, R.L. and Benkovic, S.J., eds.) Vol 1. pp.255-306. Wiley, New York.

MALEY, F. and MALEY, G.F. 1990. A tale of two enzymes, Deoxycytidylate deaminase and thymidylate synthase. Progress in Nucleic Acid Research and

Molecular Biology. 39:49-80.

MASON, J. Intestinal transport of monoglutamyl folates in mammalian systems. 1990. in *Folic Acid Metabolism in Health and Disease*. (Picciano, M.F., Stokstad, E.L.R. and Gregory, J.F. III eds.) pp. 47-63. Wiley, New York.

McGUIRE, J.J. and COWARD, J.K. 1984. Pteroylpolyglutamates: biosynthesis, degradation, and function. in *Folates and Pterins*. (Blakley, R.L. and Benkovic, S.J., eds.) Vol 1. pp.135-190. Wiley, New York.

McTIGUE, M.A., DAVIS, J.F.II., KAUFMAN, B.T. and KRAUT, J. 1992. Crystal structure of chicken liver dihydrofolate reductase complexed with NADP and biopterin. Biochemistry. 31:7264-7273.

NAUGHTON, C.A., CHANDLER, C.J., DUPLANTIER, R.B. and HALSTED, C.H. 1989. Folate absorption in alcoholic pigs: in vitro hydrolysis and transport at the intestinal brush border membrane. American Journal of Clinical Nutrition. 50:1436-1441.

NEUBURGER, M., JOURDAIN, A. and DOUCE, R. 1991. Isolation of H-protein loaded with methylamine as a transient species in glycine decarboxylase reactions. Biochemical Journal 278:765-770.

NOUR, J.M. and RABINOWITZ, J.C. 1988. Purification and structural organization of 10-formyl tetrahydrofolate synthetase from spinach leaves. Journal of Cell Biology. 107: 179a.

NOUR, J.M. and RABINOWITZ, J.C. 1991. Isolation, characterization,and structural organization of 10-formyl tetrahydrofolate synthetase from spinach leaves. The Journal of Biological Chemistry. 266:18363-18369.

NOUR, J.M. and RABINOWITZ, J.C. 1992. Isolation and sequencing of the cDNA coding for spinach 10-formyl tetrahydrofolate synthetase, comparisons with the yeast, mammalian, and bacterial proteins. The Journal of Biological Chemistry. 267:16292-16296.

O'CONNOR, B.M., ROTUNDO, R.F., NIMEC, Z, McGUIRE, J.J. and GALIVAN, J. 1991. Secretion of gamma-glutamyl hydrolase in vitro. Cancer Research. 51:3874-3881.

PAUKERT, J.L., STRAUS, L.D. and RABINOWITZ, J.C. 1976. Formyl-methenyl-methylenetetrahydrofolate synthetase (combined), an ovine protein with multiple catalytic activities. The Journal of Biological Chemistry. 251:5104-5111.

PIYATHILAKE, C.J., HINE, R.J., DASANAYAKE, A.P., RICHARDS, E.W., FREEBERG, L.E., VAUGHN, V.H. and KRUMDIECK, C.L. 1992. Effect of smoking on folate levels in buccal mucosal cells. International Journal of Cancer. 52:566-569.

PRIEST, D.G., VERONEE, C.D., MANGUM, M., BEDNAREK, J.M. and DOIG, M.T. 1982. Comparison of folylpolyglutamate hydrolases of mouse liver, kidney, muscle and brain. Molecular and Cellular Biochemistry. 43:81-87.

PRISTUPA, Z.B., McAULEY, C.M. and SCRIMGEOUR, K.G. 1991. Folylpolyglutamate synthetase from beef liver: purification and some properties. Biochemical and Cellular Biology. 69:556-560.

REISENAUER, A.M. and HALSTED, C.H. 1981. Human jejunal brush border folate conjugase: Characteristics and inhibition by salicylazosulfapyridine. Biochimica et Biophysica Acta. 659:62-69.

REISENAUER, A.M., KRUMDIECK, C.L. and HALSTED, C.H. 1977. Folate conjugase: Two separate activities in human jejunum. Science 198:196-197.

RHEE, M.S., WANG, Y., NAIR, M.G. and GALIVAN, J. 1993. Acquisition of resistance to antifolates caused by enhanced gamma-glutamyl hydrolase activity. Cancer Research 53:2227-2230.

ROOS, A.J. and COSSINS, E.A. 1971. Pteroylpolyglutamate derivatives in *Pisum sativum* L., Biosynthesis of cotyledonary tetrahydropteroylglutamate during germination. Biochemical Journal. 125:17-26.

ROSOWSKY, A., GALIVAN, J., BEARDSLEY, G.P., BADER, H., O'CONNER, B.M., RUSSELLO, O., MOROSON, B.A., DEYARMAN, M.T., DERWAR, S.S. and FREISHEIM., J.H. 1992. Biochemical and biological studies on 2-desmin-2-methylaminopterin, an antifolate the polyglutamates of which are more potent inhibitors than the monoglutamate against three key enzymes of folate metabolism. Cancer Research 52:2148-2155.

ROSS, J., GREEN, J., BAUGH, C.M., MacKENZIE, R.E. and MATTHEWS, R.G. 1984. Studies on the polyglutamate specificity of methylenetetrahydrofolate dehydrogenase from pig liver. Biochemistry 23:1796-1801.

ROZEN, R., BARTON, D., DU, J., HUM, D.W., MacKENZIE, R.E. and FRANCKE, U. 1989. Chromosomal localization of the gene for the human trifunctional enzyme, methylenetetrahydrofolate dehydrogenase-

methenyltetrahydrofolate cyclohydrolase-formyltetrahydrofolate synthetase. American Journal of Human Genetics. 44:781-786.

SCHIRCH, L. 1978. Formyl-methenyl-methylenetetrahydrofolate synthetase from rabit liver (combined). Archives of Biochemistry and Biophysics 189:283-290.

SCHIRCH, L. 1984. Folates in serine and glycine metabolism. in *Folates and Pterins*. (Blakley, R.L. and Benkovic, S.J., eds.) Vol 1. pp.399-431. Wiley, New York.

SCHIRCH, V. and STRONG, W.B. 1989. Interaction of folylpolyglutamates with enzymes in one-carbon metabotism. Archieves of Biochemistry and Biophysics 269:371-380.

SHANE, B. 1986. Identification of folylpoly(gamma-glutamate) chain length by cleavage to and separation of p-aminobenzoylpoly(gamma-glutamates). in *Methods in Enzymology* (Colowick, S.P. and Kaplan, N.O., eds.) Vol 122. pp.323-330.

SHANE, B. 1989. Folylpolyglultamate synthesis and role in the regulation of one-carbon metabolism. Vitamins and Hormones. 45:263-335.

SHANE, B., LOWE, K., OSBORNE, C. KIM, J. and LIN, B. 1990. role of folyl-polyglutamate synthetase in the regulation of folate and antifolate metabolism. in *Chemistry and Biology of Pteridines 1989.* (Curtius, H. -C., Ghisla, S., and Blau, N. eds) pp. 891-895. Walter de Gruyter. New York.

SHANE, B. and STOKSTAD, E.L.R. 1984. Folates in the synthesis and catabolism of histidine. in *Folates and Pterins.* (Blakley, R.L. and Benkovic, S.J. eds.) Vol 1. pp. 433-455. Wiley, New York.

SHANNON, K.W. and RABINOWITZ, J.C. 1988. Isolation and characterization of the *Saccharomyces cerevisiae* MIS1 gene encoding mitochondrial C_1-tetra-hydrofolate synthase. The Journal of Biolgical Chemistry 263:7717-7725.

SIKORA, E. KAMINSKA, B, and GRZELAKOWSKA-SZTABET, B. 1992. Proliferation-dependency of gamma-glutamyl hydrolase activity in various mouse cells. Cell Biology International Reports. 16:369-375.

SILINK, M., REDDEL, R., BETHEL, M. and ROWE, P.B. 1975. Gamma-Glutamyl hydrolase (conjugase), purification and properties of the bovine hepatic enzyme. The Journal of Biolgical Chemistry 250:5982-5994.

SMITH, J.M. and DAUM, H.A. III. 1987. Identification and nucleotide sequence of a

gene encoding 5'-phospho-ribosylglycinamide transformylase in *Escherichia coli* K12. Journal of Biological Chemistry. 262:10565-10569.

STABEN, C. and RABINOWITZ, J.C. 1984. Formation of formylmethionyl-tRNA and initiation of protein synthesis. in *Folates and Pterins.* (Blakley, R.L. and Benkovic, S.J. eds.) Vol 1. pp. 457-495. Wiley, New York.

STOVER, P. and SCHIRCH, V. 1992. Synthesis of (6S)-5-formyl-tetrahydro-pteroyl-polyglutamates and interconversion to other reduced pteroylpolyglutamate derivatives. Analytical Biochemistry. 202:82-88.

SUR, P., PRIEST, D.G. and DOIG, M.T. 1986. Effects of growth rate and methotrexate on folate polyglutamates and folylpolyglutamate hydrolase activity in Krebs ascites cells. Biochemical and Cellular Biology. 64:363-367.

TAMURA, T., BUEHRING, K.V. and STOKSTAD, E.L.R. 1972. Enzymatic hydrolysis of pteroylpolyglutamates in cabbage, Proceedings, the Society of Experimental Biology and Medicine. 141:1022-1025.

TAMURA, T. and KAISER, L.L. 1991. The absorption of pteroylpolyglutamate and intestinal pteroylpoly-gamma-glutamyl hydrolase activity in zinc-deficient rats. The Journal of Nutrition. 121:1042-1046.

TAN, L.V.L., DRURY, E.J. and MacKENZIE, R.E. 1977. Methylenetetrahydrofolate dehydrogenase-methenyltetra-hydrofolate cyclohydrolase-formyltetrahydrofolate synthetase: A multifunctional protein from porcine liver. The Journal of Biological Chemistry 252:1117-1122.

TOLBERT, N.E. 1980. Photorespiration. in *The Biochemistry of Plants.* (Davies, D.D., ed.) Vol 2. pp.487-523. Academic Press, New York.

TURNER, S.R., IRELAND, R. and RAWSTHRONE, S. 1992a. Cloning and characterization of the P-subunit of glycine decarboxylase from pea (*Pisum sativum*). Journal of Biological Chemistry 267:5355-5360.

TURNER, S.R., IRELAND, R. and RAWSTHRONE, S. 1992b. Purification and primary amino acid sequence of the L-sbuunit of glycine decarboxylase: Evidence for a single lipoamide dehydrogenase in plant mitochondria. Journal of Biological Chemistry 267:7745-7750.

VARSHNEY, U. and RajBHANDARY, U.L. 1992. Role of methionine and formylation of initiator tRNA in initiation of protein synthesis in *Escherichia coli*. Journal of Bacteriology. 174:7819-7826.

WANG, Y., NIMEC, Z., RYAN, T., DIAS, J. and GALIVAN, J. 1993. The properties of the secreted gamma-glutamyl hydrolase from H35 hepatoma cells. Biochimica et Biophysica Acta (in press).

WANG, T.T.Y., REISENAUER, A.M. and HALSTED, C.H. 1985. Comparison of folate conjugase activities in human, pig, rat, and monkey intestine. The Journal of Nutrition 115:814-819.

WARD, G.J. and NIXON, P.F. 1990. Modulation of pteroylpolyglutamate concentration and length in response to altered folate nutrition in a comprehensive range of rat tissues. The Journal of Nutrition 120:476-484.

WASSERMAN, G.F., BENKOVIC, P.A., YOUNG, M. and BENKOVIC, S.J. 1983. Kinetic relationships between the various activities of the formyl-methenyl-methylenetetra-hydrofolate synthetase. Biochemistry. 22:1005-1013.

WEST, M.G., BARLOWE, C.K. and APPLING, D.R. 1993. Cloning and characterization of the *Saccharomyces cerevisiae* gene encoding NAD-dependent 5,10-methylenetetra-hydrofolate dehydrogenase. The Journal of Biological Chemistry. 268:153-160.

WHITEHEAD, V.M., KALMAN, T.I., ROSENBLATT, D.S., VUCHIN, M.-J. and PAYMENT, C. 1988. Regulation of methotrexate polyglutamate (MTXPG) formation in human leukemic cells. Proccedings, American Association of Cancer Research 29:287.

WHITEHEAD, V.M., KALMAN, T.I. and VUCHIN, M.-J. 1987. Inhibition of gamma-glutamyl hydrolases in human cells by 2-mercaptomethylglutaric acid. Biochemical and Biophysical Research Communications 144:292-297.

WHITEHEAD, V.M. and ROSENBLATT, D.S. 1985. Metabolism of methotrexate (MTX) and folates in transformed human lymphocytes. Proceedings, American Association of Cancer Research. 26:232.

WHITEHEAD, V.M., VUCHIN, M.-J., PAYMENT, C., HUCAL, S. and KALMAN, T.I. 1990. Study of methotrexate polyglutamate formation in cultured leukemic cells. in *Chemistry and Biology of Pteridines 1989*. (Curtius, H.-Ch., Ghisla,S. and Bau, N. eds.) pp.1166-1169. Walter de Gruyter, Berlin.

WOHLFARTH, G., GEERLINGS, G. and DIEKERT, G. 1990. Purification and properties of a NADH-dependent 5,10-methylenetetrahydrofolate reductase from *Peptostreptococcus productus*. European Journal of Biochemistry. 192-

411-417.

ZHENG, L.-L., LIN, Y., LIN, S. and COSSINS, E.A. 1992. The polyglutamate nature of plant folates. Phytochemistry 31:2277-2282.

ZHOU, J., KANG, S.S., WONG, P.W., FOURNIER, B. and ROZEN, R. 1990. Purification and characterization of methylenetetrahydrofolate reductase from hyman cadaver liver. Biochemical Medicine and Metabolic Biology. 43:234-242.